Replacement of Neutral Lipids of Low Density Lipoprotein with Esters of Long Chain Unsaturated Fatty Acids

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A method has recently been described by which the neutral lipids of plasma low density lipoprotein (LDL) can be extracted with heptane and replaced with exogenous cholesteryl esters. In the current studies we show that, in addition to cholesteryl esters, other esters of long chain fatty acids, including triacylglycerols and methyl esters, can be used to reconstitute the core of heptane-extracted LDL. For each of these classes of esters, the common structural requirement for substantial incorporation into LDL was the presence of at least one cis-double bond in the fatty acyl chain. For example, cholesteryl oleate, triolein, and methyl oleate could each be incorporated into LDL to yield a final lipid to protein mass ratio that was greater than one. In contrast, only trace amounts of esters of saturated fatty acids, such as cholesteryl stearate, tristearin, or methyl stearate, could be incorporated into LDL despite the use of a variety of solvents and different temperatures of incubation. Incorporation of these saturated compounds was not enhanced by the inclusion of unsaturated cholesteryl esters or unsaturated triacylglycerols in the reconstitution reaction. Another class of compounds that can be incorporated into heptane-extracted LDL consists of lipids that contain a polyisoprenyl side chain, such as retinyl palmitate and ubiquinone-10. Each of the reconstituted LDL preparations retained the ability to bind to the LDL receptor of human fibroblasts and thus to deliver its respective core lipid to cells. The current data establish that plasma LDL can be made to function as a carrier for a variety of hydrophobic compounds that contain either long chain saturated fatty acyl or polyisoprenyl groups. The preferential incorporation of such compounds into LDL as compared with compounds containing long chain saturated fatty acids suggests that the protein or phospholipid component of LDL may have a specific ability to interact with long chain hydrocarbons that contain one or more double bonds.

Low density lipoprotein, the major cholesterol-carrying lipoprotein of human plasma, delivers cholesterol to cells by binding to a cell surface receptor (1). Receptor-binding is a prerequisite for the cellular uptake of the lipoprotein and its delivery to lysosomes. Cholesterol liberated from the lysosomal hydrolysis of the LDL-bound cholesteryl esters satisfies the cholesterol requirements of the cell and elicits a series of regulatory responses that control the cellular cholesterol content (2).

The cholesteryl esters of LDL, which account for approximately 50% of the mass of the lipoprotein, consist chiefly of two cis-un saturated fatty acid esters, cholesteryl linoleate [C 18:2(9c,12c)] and cholesteryl oleate [C 18:1(9c)] (3). These cholesteryl esters are believed to form an apolar core that is surrounded by the more polar constituents of the lipoprotein, including various phospholipids, small amounts of free cholesterol, and a protein called apoprotein B (3, 4). The LDL receptor on cell surfaces recognizes the apoprotein B component of the lipoprotein (1, 5, 6).

We recently demonstrated that the endogenous cholesteryl esters of LDL could be extracted with heptane and replaced with exogenous cholesteryl linoleate (7). Inasmuch as the resulting reconstituted LDL particle contained its original complement of phospholipid and protein, it retained the ability to bind to the LDL receptor in cultured human fibroblasts. As a consequence of this binding, the reconstituted LDL was taken up by the cells and the cholesterol released from the lysosomal hydrolysis of the cholesteryl linoleate elicited the same regulatory actions that occur when native LDL is incubated with fibroblasts (7).

The ability to replace the cholesteryl esters of LDL while retaining the functional activity of the LDL particle raised the possibility that molecules other than cholesteryl esters might be introduced into the LDL particle and hence delivered to cells via the LDL receptor. In an initial series of studies along these lines, we incorporated 25-hydroxycholesteryl oleate into LDL and showed that uptake of this reconstituted lipoprotein by fibroblasts was receptor-dependent (8). Hydrolysis of the 25-hydroxycholesteryl oleate in lysosomes suppressed the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-controlling enzyme of cholesterol synthesis, and thereby inhibited the growth of the cells (8).

In the current experiments, we have begun to define the physical and chemical properties that determine whether a given compound can be incorporated into LDL by means of the reconstitution technique. The data indicate that in addition to cholesteryl esters a variety of other hydrophobic esters of fatty acids, including triacylglycerols and methyl esters, can replace the native cholesteryl esters in reconstituted LDL. The structural feature that facilitates the incorporation of

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these fatty acid esters into LDL is the presence of at least one double bond in the fatty acyl chain.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cholesteryl oleate and cholesteryl linoleate were purchased from Applied Science Laboratories, Inc. All other cholesteryl esters, fatty acid methyl esters, triacylglycerols, and linoleyl alcohol were purchased from Nu Chek Prep, Inc. [1,2-3H]Cholesterol (43 Ci/mmol) and sodium [32P]orthophosphate were obtained from American/ Searle. [1,2-3H]cholesterol linoleate was synthesized from [1,2-3H]cholesterol and linoleyl chloride as previously described (7). Retinyl palmitate and ubiquinone-10 were purchased from Sigma Chemical Co. 3-Hydroxy-3-methyl[1-14C]glutaryl-CoA (49.5 μCi/mmol) and Aquasol were purchased from New England Nuclear Corp. Tissue culture supplies and chemical reagents were obtained from previously described sources (7, 9).

**Preparation of Reconstituted LDL**

Reconstituted LDL was prepared by one of two methods.

**Method 1**—This method was a minor modification of the procedure that was previously described (7). Unlabeled LDL or [1,2-3H]LDL was dialyzed against 0.3 mM sodium EDTA (pH 7) (Step 1). Aliquots of dialyzed LDL (0.7 or 1.9 mg of protein) were lyophilized in Siliclad-treated glass tubes (13 × 100 mm) (Step 2) and more than 99% of the neutral lipids were removed by two extractions with 5 ml of heptane at −10°C (Step 3). Steps 1 to 3 are based on the extraction procedure originally described by Gustafson (11). The heptane-extracted LDL was then reconstituted by addition of 206 µl of carbon tetrachloride containing 0 to 6 mg of neutral lipid, usually unlabeled cholesteryl linoleate or [1,2-3H]cholesterol linoleate (Step 4). The tubes were incubated for 1 h at −10°C (Step 5). The heptane was then evaporated under nitrogen at 0°C (Step 6). The reconstituted LDL was solubilized by incubation in either 300 µl (0.7 mg of protein sample) or 1 ml (1.9 mg of protein sample) of 0.3 mM Tricine (pH 8.4) for 12 h at 0°C (Step 7), after which the starch and unincorporated neutral lipid were removed by centrifugation as previously described (Step 8) (7).

**Method 2**—This method differs from Method 1 in two respects: the solvent used in Step 4 and the temperature used in Steps 5 and 6. Aliquots of dialyzed LDL were lyophilized in the presence of potato starch and the neutral lipids were removed by heptane extraction as in Method 1 (Steps 1 to 3). The heptane-extracted LDL was reconstituted in Step 4 by addition of 200 µl of heptane containing 0 to 6 mg of neutral lipid, usually unlabeled cholesteryl linoleate or [1,2-3H]cholesterol linoleate (Step 4). The tubes were incubated for 1 h at −10°C (Step 5). The heptane was then evaporated under nitrogen at 0°C (Step 6). The reconstituted LDL was solubilized by incubation in either 300 µl (0.7 mg of protein sample) or 1 ml (1.9 mg of protein sample) of 0.3 mM Tricine (pH 7.4) for 12 h at 0°C (Step 7), after which the starch and unincorporated neutral lipid were removed by centrifugation as previously described (Step 8) (7).

**RESULTS**

In the previously described reconstitution procedure, LDL is adsorbed onto starch and its neutral lipids are extracted with heptane (11). Exogenous cholesteryl esters in heptane solution are then added to the LDL-starch residue. The heptane carrier is evaporated and the exogenous cholesteryl esters are deposited in association with LDL residue on the starch (7). To determine whether the evaporation step is required for reconstitution, we performed an experiment in which the heptane-extracted LDL was incubated for 1 h with increasing amounts of [1,2-3H]cholesterol linoleate dissolved in heptane (Fig. 1). The samples were then treated in one of two ways: in one group of samples the heptane was evaporated by the standard procedure and in the second group of samples the heptane-cholesterol linoleate solution was removed by aspiration without any evaporation. Both groups of lipoproteins were then solubilized in an aqueous buffer. Fig. 1A shows the amount of LDL-protein recovered in the final aqueous supernatant. When the heptane-cholesterol linoleate solution was aspirated without evaporation, approximately 65% of the initial protein

Chen et al. (16). The content of methyl linolate and linoleyl alcohol in reconstituted LDL was measured by gas-liquid chromatography after extracting the lipids by the method of Folch et al. (15). Methyl stearate and stearyl alcohol were used as internal standards to correct for procedural losses. The content of triacylglycerols in native and reconstituted LDL was determined by the enzymatic assay of Schmidt and von Dahl (17) using Boehringer Mannheim triacylglycerol assay reagents (Reagent Set 126012) as previously described (18). The content of retinyl palmitate and ubiquinone-10 in reconstituted LDL was determined by extracting the neutral lipid from the lipoprotein (20 to 70 μg of protein) by the method of Folch et al. (15), dissolving the lipid in ethanol, and comparing its absorbance at 526 nm (retinyl palmitate) or 470 nm (ubiquinone-10) with the absorbance of authentic samples of the appropriate lipid (0 to 100 μg), which had been subjected to a similar extraction procedure.

**Cells**

Cultured fibroblasts were derived from skin biopsies obtained from a normal subject and from a patient with the receptor-negative form of homozygous familial hypercholesterolemia (2). Cells were grown in monolayer, used between the 5th and 20th passage, and maintained in a humidified incubator (5% CO2 at 37°C in 75 cm2) when subjected to electrophoresis in agarose gels at pH 8.6 (7).

**Measurement of Chemical Composition of Native and Reconstituted LDL**

The protein content of lipoproteins was determined by the method of Lowry et al. (12) using bovine serum albumin as a standard. The total content of phosphate was measured by the method of Schmid and von Dahl (17) using Boehringer Mannheim triacylglycerol assay reagents (Reagent Set 126012) as previously described (18). Measurement of chemical composition of native and reconstituted LDL when subjected to electrophoresis in agarose gels at pH 8.6 (7). Moreover, reconstituted LDL prepared by Methods 1 and 2 were shown to have the same electrophoretic mobility as that of native LDL when subjected to electrophoresis in agarose gels at pH 8.6 (7).
was recovered, and the recovery was not dependent on the presence of cholesteryl linoleate in the heptane solution. On the other hand, when the heptane was evaporated, the recovery of protein was strictly dependent on the presence of cholesteryl linoleate in the solution. In the absence of cholesteryl linoleate, less than 10% of the protein could be solubilized. On the other hand, when the heptane was not evaporated, the ratio of cholesteryl linoleate to protein was low at all cholesteryl linoleate concentrations. The data of Fig. 1 established three points about the reconstitution procedure: 1) evaporation of the solvent is required in order to introduce substantial amounts of exogenous cholesteryl linoleate into LDL; 2) when the heptane is evaporated in the absence of cholesteryl linoleate, most of the protein is denatured and cannot be solubilized; and 3) when cholesteryl linoleate is present during the evaporation, cholesteryl linoleate is incorporated and the lipoprotein can be recovered in a water soluble form.

To determine whether the use of heptane was critical in the evaporation step of the reconstitution, we conducted an experiment in which the neutral lipids of LDL were extracted with heptane, and exogenous [3H]cholesteryl linoleate was then added in one of several solvents, each of which was subsequently removed by evaporation. The data in Table I are arranged in order of increasing dielectric constants for the solvents. The relatively nonpolar solvents, including heptane, petroleum ether, carbon tetrachloride, benzene, and diethyl ether, gave equivalent yields of protein in the reconstitution. In all cases, the ratio of [3H]cholesteryl linoleate to protein in the reconstituted LDL was similar. On the other hand, more polar solvents, such as chloroform and acetone, gave considerably lower yields of protein. Although the yield of protein dropped with the use of these more polar solvents, the mass ratio of [3H]cholesteryl linoleate to protein in the small amount of water-soluble material that was recovered was similar to that obtained using heptane.

Each of the reconstituted lipoprotein particles described in Table I was also tested for its ability to bind to the LDL receptor and to be taken up and hydrolyzed by fibroblasts. These experiments were performed by incubating the reconstituted lipoproteins with fibroblast monolayers at a concentration of 10 pg of protein/ml and measuring the amount of [3H]cholesteryl linoleate hydrolyzed after 6 h. The data show that all of the recovered lipoproteins were biologically active. Even in the case of the chloroform- or acetone-treated materials, in which the amounts of lipoprotein recovered were low, the lipoprotein that was recovered in the aqueous supernatant retained biologic activity.

To determine the effect of the fatty acid moiety of the cholesteryl ester on the reconstitution reaction, we incubated heptane-extracted LDL with cholesteryl esters of saturated and cis-monounsaturated fatty acids of varying chain length. Carbon tetrachlorid was employed as a solvent for these reconstitutions because the cholesteryl esters of long chain saturated fatty acids were more soluble in this solvent than in heptane. As shown in Fig. 2A, cholesteryl esters of saturated fatty acids with chain length less than 14 carbons partially protected the lipoprotein against denaturation during the solvent evaporation step and hence allowed the recovery of protein in the aqueous supernatant. On the other hand, cho-
Aliquots of dialyzed LDL (1.9 mg of protein) were lyophilized in the presence of potato starch, and the neutral lipids were removed by heptane extraction. Aliquots of [3H]-cholesteryl linoleate (6 mg, 3.7 \times 10^7 cpm/mg) in heptane were evaporated under nitrogen, and the [3H]-cholesteryl linoleate was resuspended in 200 \mu l of the indicated solvent at room temperature. Each mixture was cooled to -10°C and then added to the heptane-extracted LDL-starch residue. After incubation for 1 h at -10°C, the solvent was evaporated at 0°C and the reconstituted lipoproteins were solubilized by incubation in 1 ml of 10 mM Tricine (pH 8.4). The amounts of protein and [3H]-cholesteryl linoleate recovered in the reconstituted LDL were determined as described under “Experimental Procedures.” Each preparation of reconstituted LDL was incubated with fibroblasts (Day 7) at a concentration of 10 \mu g of protein/ml in 2 ml of growth medium containing 10% lipoprotein-deficient serum in the absence and presence of 500 \mu g of protein/ml of unlabeled native LDL. After incubation for 6 h at 37°C, the amount of [3H]-cholesteryl linoleate hydrolyzed was determined as described under “Experimental Procedures.” The high affinity values represent the difference between the value obtained in the absence of unlabeled LDL (total value) and the value obtained in the presence of excess unlabeled LDL. Each value is the average of duplicate incubations.

| Solvent          | Protein Recovered | Cholesterol Linoleate Recovered | Mass Ratio | Total | Affinity |
|------------------|-------------------|--------------------------------|------------|-------|----------|
| Heptane (1.9)    | 0.82              | 1.5                            | 1.8        | 12.1  | 10.9     |
| Petroleum ether  | 0.82              | 1.5                            | 1.8        | 10.3  | 9.3      |
| Carbon tetrachloride (2.2) | 0.78          | 1.3                            | 1.7        | 9.7   | 8.6      |
| Benzene (2.3)    | 0.78              | 1.3                            | 1.7        | 9.5   | 8.4      |
| Diethyl ether (4.3) | 0.74          | 1.3                            | 1.7        | 9.8   | 8.6      |
| Chloroform (4.8) | 0.29              | 0.5                            | 1.8        | 12.5  | 11.1     |
| Acetone (20.7)   | 0.28              | 0.5                            | 1.8        | 9.9   | 8.3      |
| Chloroform/methanol (2:1) | 0.05       | 0.05                           | 1.0        | 0.5   | 0.5      |

*The value in parentheses represents the dielectric constant for the indicated solvent (20) except for the value for heptane. Its dielectric constant is assumed to be the same as the published values for hexane and octane, which are both 1.9 (20). The difference in behavior of the saturated and unsaturated fatty acids was also observed in experiments comparing cholesteryl esters of 18 carbon fatty acids that possess varying numbers and configurations of double bonds (Fig. 3). These reconstitution experiments were performed in two different solvent systems and at two different temperatures: heptane at 0°C (Method 1, Fig. 3A) and carbon tetrachloride at room temperature (Method 2, Fig. 3B). With Method 1, there was no detectable incorporation of cholesteryl oleate (C 18:0) into LDL, and less than 10% of the added protein was recovered in the aqueous supernatant. When cholesteryl olate [C 18:1(9c)] was used for reconstitution, the protein yield was increased to 40%, and large amounts of cholesteryl olate were incorporated into the lipoprotein. Cholesteryl linoleate [C 18:2 (9c, 12c)] and cholesteryl linolenate [C 18:3(9c, 12c, 15c)] were incorporated into LDL with yields that were similar to that of cholesteryl oleate (Fig. 3A). In the above experiments the time of incubation of the heptane-extracted LDL with the exogenous lipid prior to evaporation (Step 5) was 1 h. In other experiments, we found that shortening the incubation time to 30 s did not affect the final yield of lipid and protein in LDL reconstituted with cholesteryl linoleate.

Quantitatively similar results were obtained when carbon tetrachloride was used as the solvent (Method 2, Fig. 3B). Cholesteryl stearate and cholesteryl olate gave protein recoveries of 10% and 54%, respectively. In this experiment, we
Reconstitution of Low Density Lipoprotein

Fig. 3. Reconstitution of heptane-extracted LDL with cholesteryl esters of the C 18 series: effect of the number of double bonds and their configuration. Aliquots of dialyzed LDL (A, 1.9 mg of protein; B, 0.7 mg of protein) were lyophilized in the presence of potato starch, the neutral lipids were removed by heptane extraction, and the lipoproteins were reconstituted by either Method 1 (A) or Method 2 (B) with the indicated cholesteryl esters (6 mg of cholesteryl ester in 200 μl of heptane and 4 mg of cholesteryl ester in 133 μl of carbon tetrachloride for Method 1 and Method 2, respectively) as described under "Experimental Procedures." The amounts of protein (solid bars) and cholesteryl ester (striped bars) recovered in the reconstituted LDL were determined as described under "Experimental Procedures." In a control tube in which heptane-extracted LDL was reconstituted with either heptane or carbon tetrachloride containing no cholesteryl esters, 0.06 mg of protein and no cholesteryl esters were recovered in the aqueous supernatant. Each value represents either a single reconstitution (A) or the average of duplicate reconstitutions (B).

Fig. 4. Suppression of HMG-CoA reductase activity in human fibroblasts by native LDL (●—●) and by heptane-extracted LDL reconstituted with cholesteryl linoleate ( ●) and cholesteryl esters containing cis-monounsaturated ( ●) and saturated ( ○) fatty acids. LDL preparations were reconstituted by either Method 1 or Method 2. The number contained within the ● or the ○ indicates the number of carbon atoms in the fatty acyl chain of the cholesteryl ester used to reconstitute the heptane-extracted LDL. Either native LDL ( ●) or one of the reconstituted LDL preparations ( ●, ○) was added. The abscissa gives the total mass of cholesteryl per ml of medium added to 1.5 ml of growth medium containing 10% lipoprotein-deficient serum. Fibroblast monolayers (Day 2 of growth) were incubated for 6 h at 37°C with each of the solutions, after which the cells were harvested for measurement of HMG-CoA reductase activity. These data represent a composite of values obtained in 11 different experiments. The HMG-CoA reductase activity is expressed as a percentage of the enzyme activity in cells that received no lipoproteins in the same experiment. The "100% control values" varied from 126 to 353 (mean = 229) pmol min⁻¹ mg protein⁻¹.
detected in the aqueous supernatant of any of the samples incubated with cholesteryl stearate. The lipoproteins recovered in various reconstitution experiments as described above were tested for biologic activity by measuring their ability to suppress HMG-CoA reductase activity in monolayers of growing human fibroblasts (Fig. 4). The requirement for unsaturated fatty acids in the reconstitution of LDL also applied when methyl esters of fatty acids were used to reconstitute LDL. Thus, methyl palmitate and methyl stearate, two saturated fatty acid esters, were not incorporated into LDL, whereas the unsaturated fatty acid esters methyl palmitoleate and methyl linoleate were incorporated in amounts that were similar to those for cholesteryl linoleate (data not shown). Fig. 6 shows the incorporation of methyl linoleate into LDL as a function of the amount of lipid added to the heptane-extracted LDL. The protein recovery (Fig. 6A) and the mass ratio of lipid to protein (Fig. 6C) were

**Fig. 5 (left).** Reconstitution of heptane-extracted LDL with triacylglycerols containing saturated (○, △) and unsaturated (△, ▲) fatty acids. Aliquots of dialyzed LDL (0.7 mg of protein) were lyophilized in the presence of potato starch, the neutral lipids were removed by heptane extraction, and each sample was reconstituted with the indicated amount of trilinolein (△), triolein (○), tripalmitin (●), tristearin (▲), or cholesteryl linoleate (X) according to Method 2 under "Experimental Procedures." The amounts of protein (A) and lipid (B) recovered in the reconstituted LDL were determined as described under "Experimental Procedures." The mass ratio of neutral lipid to protein is shown in C. Each value represents the average of duplicate reconstitutions.

**Fig. 6 (center).** Reconstitution of heptane-extracted LDL with methyl linoleate (●) and linoleyl alcohol (●). Aliquots of dialyzed LDL (0.7 mg of protein) were lyophilized in the presence of potato starch, the neutral lipids were removed by heptane extraction, and each sample was reconstituted with the indicated amount of methyl linoleate (●) or linoleyl alcohol (●) according to Method 2 under "Experimental Procedures." The amounts of protein (A) and lipid (B) recovered in the reconstituted LDL were determined as described under "Experimental Procedures." The mass ratio of neutral lipid to protein is shown in C. Each value represents the average of duplicate reconstitutions.

**Fig. 7 (right).** Failure of cholesteryl linoleate to enhance incorporation of cholesteryl stearate into heptane-extracted LDL. Aliquots of dialyzed LDL (0.7 mg of protein) were lyophilized in the presence of potato starch and the neutral lipids were removed by heptane extraction. Samples of heptane-extracted LDL were reconstituted with the indicated mixture of [3H]cholesteryl linoleate (2 × 10^6 cpm/mg) (△) and cholesteryl stearate (●) in 133 μl of carbon tetrachloride according to Method 2 under "Experimental Procedures." The amounts of protein (A), [3H]cholesteryl linoleate (B), and total cholesteryl esters recovered in the reconstituted LDL were determined as described under "Experimental Procedures." The amount of cholesteryl stearate recovered in the reconstituted LDL (B) was calculated by subtracting the amount of [3H]cholesteryl linoleate recovered from the total amount of cholesteryl esters recovered. In a control tube in which heptane-extracted LDL was reconstituted with carbon tetrachloride containing no cholesteryl esters, 0.01 mg of protein and no cholesteryl esters were recovered in the aqueous supernatant. Each value represents a single reconstitution.
similar to values obtained in other experiments with cholesterol linoleate (cf. Fig. 5). The data in Fig. 6 also show that appreciable amounts of linoleyl alcohol could be incorporated into LDL. Two experiments were performed to determine whether the presence of an unsaturated fatty acid ester would enhance the incorporation of a saturated fatty acid ester into LDL. In the first experiment shown in Fig. 7, samples of heptane-extracted LDL were incubated with a total of 4 mg of cholesterol ester that was made up of varying proportions of [3H]cholesterol linoleate and unlabeled cholesterol stearate. When the tubes contained only cholesterol stearate, the recovery of protein was low (Fig. 7A) and no cholesterol ester was recovered in the aqueous supernatant (Fig. 7B). As the proportion of cholesterol linoleate was increased, the yield of protein increased. All of the cholesterol esters in the reconstituted LDL could be accounted for by the measured amount of [3H]cholesterol linoleate. No cholesteryl stearate was recovered in the aqueous supernatant (Fig. 7). In the second experiment, the tubes also contained retinyl palmitate and ubiquinone-10. When the tubes contained only cholesterol stearate, the recovery of protein was low (Fig. 7A) and no cholesteryl stearate was recovered in any of the aqueous supernatants (Fig. 7B).

In other mixing experiments, we attempted to reconstitute LDL with cholesterol stearate (4 mg/tube) by incorporating it in the presence of increasing amounts of each of two unsaturated triacylglycerols, triolein and trilinolein. As expected from the previous data, reconstitution using cholesterol stearate alone resulted in little protein and no measurable cholesteryl ester in the aqueous supernatant. The presence of either triolein or trilinolein at 2 mg/tube increased the yield of protein by 5- to 8-fold, but only trace amounts of cholesteryl stearate were detected in the reconstituted LDL.

As previously demonstrated, LDL reconstituted with cholesterol linoleate was able to suppress HMG-CoA reductase activity in human fibroblasts in a manner similar to that of native LDL (7, 8). On the other hand, LDL reconstituted with methyl linoleate or with trilinolein did not suppress HMG-CoA reductase activity (data not shown) even though these reconstituted lipoproteins were taken up and hydrolyzed by the cells at rates that were similar to those of native LDL (see below). These data are in keeping with earlier studies indicating that the cholesterol of LDL is the component responsible for lipoprotein-mediated suppression of HMG-CoA reductase activity (2, 6, 21).

In addition to compounds that contain unsaturated fatty acids, another class of compounds that could be incorporated into LDL, were those that contained polyisoprenyl chains. Table II shows that retinyl palmitate, a polyisoprenyl alcohol ester of a saturated fatty acid was incorporated into LDL in an amount similar to that obtained with cholesterol linoleate. Ubiquinone-10, another molecule with a polyisoprenyl side chain, could also be incorporated into LDL in appreciable amounts (Table II). We have also been able to reconstitute heptane-extracted LDL with retinol and chlorophyll, both of which contain a polyisoprenyl side chain (data not shown).

To test the biologic activity of the LDL preparations that were reconstituted with molecules other than cholesterol esters, we carried out the reconstitution procedure with 125I-LDL. The resulting 125I-labeled reconstituted LDL preparations were then incubated with normal human fibroblasts and the amount of 125I-labeled protein that was degraded was measured (Table III). To demonstrate that the degradation was mediated by the LDL receptor, each of the 125I-labeled reconstituted LDL preparations was also incubated with cells from a patient with homozygous familial hypercholesterolemia. These mutant cells lack LDL receptors and hence are unable to take up and degrade 125I-LDL through the LDL receptor pathway (1, 2). The data in Table III show that preparations of 125I-LDL reconstituted with cholesterol linoleate, trilinolein, methyl linoleate, and retinyl palmitate were each able to bind to the LDL receptor and be delivered to lysosomes, as indicated by their normal rates of degradation in normal fibroblasts. In each case, the degradation was markedly reduced in the homozygous familial hypercholesterolemia cells.

**TABLE II**

| Lipid added | Amount of lipid added (a) mg/tube | Reconstituted LDL recovered | Protein (b) mg/tube | Lipid (b) mg/tube | Mass ratio (b)/mg (a) | Experiment A. Retinyl palmitate | 0 | 0.37 | 0 | 0 | 0 | 3.5 | 1.06 | 1.29 | 1.21 | 20 | 1.71 | 2.53 | 1.48 |
|-------------|----------------------------------|-----------------------------|-------------------|------------------|-------------------|----------------------|----|------|----|------|----|------|------|------|------|----|------|------|------|
| Experiment B. Ubiquinone-10 | 0 | 0.24 | 0 | 0 | 1 | 0.41 | 0.34 | 0.05 | 4 | 0.50 | 0.64 | 1.28 | 13 | 0.39 | 0.31 | 0.79 |

**TABLE III**

Degradation of various preparations of 125I-labeled LDL by normal fibroblasts and mutant fibroblasts lacking functional LDL receptors

Aliquots of dialyzed 125I-LDL (0.7 mg of protein, 24 cpm/ng of protein) were lyophilized in the presence of potato starch, the neutral lipids were removed by heptane extraction, and the samples were reconstituted with 4 mg of the indicated lipid according to Method 1 under "Experimental Procedures." Each preparation of reconstituted LDL and native LDL (24 cpm/ng of protein) was incubated with monolayers of normal or mutant (familial hypercholesterolemia homozygote) fibroblasts (day 7) at a concentration of 9 μg of protein/ml in 2 ml of growth medium containing 10% lipoprotein-deficient serum in the absence or presence of 500 μg of protein/ml of unlabeled LDL. After incubation for 5 h at 37°C, the amount of high affinity 125I-LDL degradation was determined as described under "Experimental Procedures." Each value represents the average of duplicate incubations. The high affinity values represent the difference between the value obtained in the absence of unlabeled LDL (total degradation) and the value obtained in the presence of excess unlabeled LDL. The high affinity degradation was more than 90% of the total degradation for all LDL preparations except r-[methyl linoleate]-LDL in which the value for high affinity degradation in the normal cells (3400) was 75% of the value for total degradation (4480).

**Preparation of 125I-labeled LDL**

| High affinity degradation of 125I-LDL | Normal cells | Mutant cells | ng 5 h⁻¹ mg⁻¹ protein⁻¹ |
|-------------------------------------|-------------|--------------|----------------------|
| Native LDL                          | 3270        | 37           |
| r-[Cholesterol linoleate]-LDL       | 3650        | 110          |
| r-[Trilinolein]-LDL                 | 3180        | 0            |
| r-[Methyl linoleate]-LDL            | 3400        | 56           |
| r-[Retinyl palmitate]-LDL           | 2720        | 0            |

**DISCUSSION**

Several conclusions can be drawn from the data in the current paper. First, the neutral lipids in the core of LDL can be replaced with a wide variety of lipid-soluble molecules whose structures differ markedly from those of the cholesterol...
esters that normally occupy this core. Second, the exogenous neutral lipids can be delivered in a variety of nonpolar solvents. Third, in order for the exogenous neutral lipid to be incorporated into LDL, it is necessary that the solvent be removed by evaporation so that the added lipid deposits in association with the heptane-extracted LDL residue. Fourth, the incorporation of esters of long chain fatty acids into LDL, including cholesteryl esters, triacylglycerols, and methyl esters, is markedly enhanced when the fatty acyl chain contains at least one double bond. Fifth, compounds containing polyisoprenyl chains can also be incorporated into LDL with high efficiency.

The inability to incorporate cholesteryl, glyceryl, and methyl esters of saturated fatty acids might theoretically have been due to two physical properties of the saturated esters that differ from those of the unsaturated esters. First, the esters of saturated fatty acids are generally somewhat less soluble in heptane than the corresponding esters of the unsaturated fatty acids. This limited heptane solubility might have limited the incorporation of these compounds into LDL by preventing them from reaching some critical concentration in the solvent during the evaporation step. This possibility seems unlikely in view of the finding that cholesteryl esters of long chain saturated fatty acids could not be incorporated into LDL even when they were dissolved in solvents such as carbon tetrachloride or benzene, two solvents in which these cholesteryl esters were much more soluble than they were in heptane. A second reason for the failure to incorporate the saturated fatty acid esters could have been due to the fact that esters of unsaturated fatty acids, but not saturated fatty acids, will form thermotropic liquid crystals or mesophases at temperatures below 60°C (22). This possibility seems unlikely in view of the fact that the evaporation step was routinely conducted at 0°C, a temperature at which both the saturated and unsaturated cholesteryl esters exist in a solid phase (92). Moreover, cholesteryl linoleate was incorporated into LDL at both 0°C and at 37°C, whereas cholesteryl stearate was not incorporated at either temperature.

Considered together, the above data suggest that neither the differences in solubility of saturated and unsaturated cholesteryl esters nor the differences in phase behavior are likely to account for the all-or-none difference in the incorporation of the saturated and unsaturated fatty acid esters into LDL. Rather, the data are more compatible with the notion that the physical characteristics of the LDL particle itself favor the incorporation of unsaturated rather than saturated fatty acid esters, thus implying that differences in the three-dimensional structures of these two classes of fatty acids are important. Consistent with this hypothesis is the observation that the cholesteryl ester of the trans-isomer of the 18-carbon monounsaturated fatty acid (elaidate) was incorporated less well than the cis-isomer (oleate), suggesting that the link in the fatty acyl chain induced by the cis-configuration about the double bond plays some role in allowing the molecule to be incorporated into LDL.

The observation that heptane-extracted LDL could be reconstituted with ubiquinone-109 and retinol, each of which contains a long polyisoprenyl chain, suggests that the polyisoprenyl group acts like the unsaturated fatty acyl group in facilitating the incorporation of lipophilic compounds into LDL. When a long chain polyisoprenyl alcohol (retinol) was attached to a long chain saturated fatty acid (palmitate), the polyisoprenyl chain appeared to compensate for the lack of a double bond in the saturated fatty acid, and the resultant ester (retinyl palmitate) was incorporated into LDL in large amounts. These data suggest that the lack of incorporation of cholesteryl, glyceryl, or methyl esters of saturated fatty acids is not due to a negative influence of the saturated fatty acyl chain per se, but rather to the lack of a positive influence contributed by the double bond. Consistent with this conclusion was the finding that the addition of cholesteryl palmitate to the reconstitution reaction in amounts up to 2.5 mg/tube did not inhibit the incorporation of cholesteryl linoleate (1 mg/tube) into heptane-extracted LDL (0.7 mg protein/tube) (data not shown).

It seems likely that the preferential incorporation into LDL of esters of long chain fatty acid containing double bonds relates to some intrinsic property of the protein or phospholipid component of LDL. It may be that one or both of these components of LDL has a specific ability to interact with long chain fatty acyl groups that contain double bonds. This possibility should be amenable to future evaluation by X-ray and neutron diffraction studies of LDL reconstituted with suitable probe molecules.

Each preparation of reconstituted LDL used in this study was characterized by determining its protein and neutral lipid content and by testing its ability to suppress HMG-CoA reductase activity in human fibroblasts. Moreover, preparations of LDL reconstituted with the various unsaturated fatty acid esters and polyisoprenyl compounds were subjected to agarose gel electrophoresis and each was shown to comigrate with native LDL. More detailed physical and biologic studies designed to further characterize the various preparations of reconstituted LDL are in progress.

The current results have one practical implication. In future attempts to incorporate molecules of biologic interest into LDL, the incorporation should be enhanced by attaching the molecules to long chain hydrocarbons containing one or more cis-double bonds.

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