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Biochemistry

Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein

(human fibroblasts/familial hypercholesterolemia/cholesterol metabolism/atherosclerosis/lysosomal acid hydrolases)

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ABSTRACT **Selective radioactive labeling of the cholesteryl esters contained within human plasma low density lipoprotein has allowed the study of the metabolism of these molecules in monolayers and extracts of cultured human fibroblasts. In monolayers of normal cells, binding of low density lipoprotein to its cell surface receptor was followed by rapid hydrolysis of the [^{3}H]cholesteryl linoleate contained within the lipoprotein and accumulation of the liberated [^{3}H]cholesterol within the cell. The stoichiometry of the degradative process suggested that the protein and cholesteryl ester components of the lipoprotein were hydrolyzed in parallel. Incubation of intact fibroblasts with chloroquine, a known inhibitor of lysosomal degradative processes, inhibited the hydrolysis of the lipoprotein-bound cholesteryl esters. Fibroblasts from subjects with the homozygous form of familial hypercholesterolemia, which lack functional low density lipoprotein receptors and thus are unable to take up this lipoprotein when it is present in the culture medium at low concentrations, were therefore unable to hydrolyze the lipoprotein-bound [^{3}H]cholesteryl linoleate. However, cell-free extracts from these mutant cells were capable of hydrolyzing the lipoprotein-bound [^{3}H]cholesteryl linoleate at the same rapid rate as normal cells when incubated at acid pH. These data illustrate the essential role of the low density lipoprotein receptor and of lysosomal acid hydrolases in the metabolic utilization of low density lipoproteins by cultured human fibroblasts.**

Studies in cultured human fibroblasts have disclosed the existence of a specific cell surface receptor that binds low density lipoprotein (LDL), the major cholesterol-carrying lipoprotein of human plasma (1–3). Binding of LDL to its receptor initiates a process by which the lipoprotein is interiorized through endocytosis, after which its protein component is hydrolyzed to its constituent amino acids in a reaction that appears to occur within lysosomes (2, *). The uptake and degradation of LDL by this mechanism leads to two important regulatory events in cholesterol metabolism: (*i*) cellular cholesterol synthesis is reduced through a suppression of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase [EC 1.1.1.34; mevalonate:NADP$^+$ oxidoreductase (CoA-acylating)] (4, 5) and (*ii*) cellular cholesteryl ester formation is enhanced through an activation of fatty acyl-CoA:cholesteryl acyltransferase (EC 2.3.1.26; acyl-CoA:cholesterol *O*-acyltransferase) (6, 7). The net result of the LDL-cellular interaction is an increase in the content of free and esterified cholesterol within the cell (8).

The finding that LDL stimulated intact fibroblasts to form cholesteryl esters was particularly striking since more than two-thirds of the cholesterol contained in LDL was already in an esterified form, largely as cholesteryl linoleate (9). However, despite the abundance of LDL-bound cholesteryl esters, all of the increase in the cellular content of cholesteryl esters that occurred in the presence of LDL could be accounted for by cholesterol newly esterified within the cell (8). These data suggested that in the course of the LDL uptake and degradation process the cholesteryl esters in LDL were hydrolyzed and the liberated free cholesterol was subsequently reesterified intracellularly (6). However, confirmation of this hypothesis by direct measurement of the fate of LDL-bound cholesteryl esters has not been possible because adequate methods for the selective radioactive labeling of cholesteryl esters in LDL have not been available.

In the present experiments we demonstrate that [^{3}H]cholesteryl linoleate can be incorporated into LDL *in vitro* in the presence of dimethylsulfoxide and that the resultant radioactive preparation retains full biologic activity. Using the preparation of [^{3}H]cholesteryl linoleate-LDL, we have shown that the cholesteryl ester component of LDL is hydrolyzed in parallel with the hydrolysis of the protein component and that both of these degradative reactions depend on the binding of LDL to its receptor.

METHODS

Cells. Cultured fibroblasts were derived from skin biopsies obtained from either normal subjects or patients with the receptor-negative form of homozygous familial hypercholesterolemia (10). All cells were grown in monolayer and used between the 5th and 20th passage. Cells were maintained in a humidified incubator (5% CO_2) at 37° in 75 cm^2 stock flasks (Falcon) containing 10 ml of growth medium consisting of Eagle's minimum essential medium (Gibco, Cat. no. F-11) supplemented with penicillin (100 units/ml); streptomycin (100 μg/ml); 20 mM Tricine-Cl, pH 7.4; 24 mM $NaHCO_3$; 1% (v/v) nonessential amino acids; and 10% (v/v) fetal calf serum (Flow Laboratories). All experiments were done in a similar format: Confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin/0.02% EDTA solution and were seeded (day 0) at a concentration of 1×10^5 cells per dish into 60 × 15 mm dishes (Falcon) containing 3 ml of growth medium with 10% fetal calf serum. On day 3 the medium was replaced with 3 ml of fresh growth medium containing 10% fetal calf serum. On day 6, when the cells were not yet confluent, each monolayer was washed with 3 ml of Dulbecco's phosphate-buffered saline, and 2 ml of fresh medium containing 5% (v/v) human lipoprotein-deficient serum (LPDS) was added (final protein concentration 2.5 mg/ml). All experiments were initiated on day 7 after the cells had been incubated for 24 hr in the presence of LPDS.

Lipoproteins. Human LDL (density 1.019–1.063 g/ml) and LPDS (density > 1.215 g/ml) were obtained from the

Abbreviations: LDL, low density lipoprotein; FH, familial hypercholesterolemia; LPDS, human lipoprotein-deficient serum; [^{3}H]CL-LDL, [^{3}H]cholesteryl linoleate bound to low density lipoprotein.

* J. L. Goldstein, G. Y. Brunschede, and M. S. Brown (1975) *J. Biol. Chem.*, in press.

plasma of healthy subjects and prepared by differential ultracentrifugation (5). In the LDL used in the present studies, the ratio of total cholesterol to protein content on a weight basis was 1.6:1, and 70% of the total cholesterol was esterified. Cholesteryl linoleate comprised 50% of the total cholesteryl esters and cholesteryl oleate comprised 20%.

Preparation of [^{3}H]Cholesteryl Linoleate-LDL. [*1,2*-^{3}H]Cholesterol (New England Nuclear Corp., 52 Ci/mmol) was reacted with linoleoyl chloride (Nu Check Prep, Inc.) in dry pyridine, and the mixture was extracted as described by Goodman (11). The resultant [^{3}H]cholesteryl linoleate was purified by thin-layer chromatography on plastic-backed sheets coated with silica gel G without gypsum (Brinkmann) developed with petroleum ether/benzene (2:1). The yield averaged 80%. A typical batch of [^{3}H]cholesteryl linoleate bound to LDL ([^{3}H]CL-LDL) was prepared in the following way: 9.3 μg (328 $\times$ 10^6 cpm) of [^{3}H]cholesteryl linoleate in chloroform/methanol (2:1) was evaporated to dryness and taken up in 0.56 ml of solution containing 0.15 M NaCl; 0.3 mM EDTA, pH 7; and 10% (v/v) dimethylsulfoxide. The mixture was incubated for 10 min at 37°, after which 10 μl of LDL (300–400 μg of protein) were added and the incubation was continued for 4 hr at 37°. The solution was then dialyzed overnight at 4° against 6 liters of buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7, after which it was centrifuged in a Beckman Microfuge (12,000 rpm, 5 min, 24°). The supernatant solution, which routinely contained 7–12% of the starting radioactivity, was stored at 4° and used for assays within 1 week. The specific activity of cholesteryl linoleate in the [^{3}H]CL-LDL was determined by extraction with chloroform/methanol (2:1) and isolation of the cholesteryl linoleate by thin-layer chromatography on 5% silver nitrate/silica gel developed with benzene. The cholesteryl linoleate was eluted from the gel, the radioactivity of one aliquot was determined in a liquid scintillation counter, and another aliquot was hydrolyzed for determination of its cholesterol content by gas-liquid chromatography (8). The content of cholesteryl linoleate measured by this method agreed closely with the value obtained by another method in which the total cholesteryl esters were isolated, subjected to methanolysis, and the content of methyl linoleate quantified by gas-liquid chromatography (12). The specific activity of multiple batches of [^{3}H]CL-LDL prepared in the above manner ranged from 130,000 to 176,900 cpm/nmol of cholesteryl linoleate. It was also determined that 1 ng of LDL protein in the [^{3}H]CL-LDL preparations was associated with about 0.56 pmol of cholesteryl linoleate. For experiments, the [^{3}H]CL-LDL was diluted with native LDL to give the final specific activity indicated in the legends.

Hydrolysis of [^{3}H]Cholesteryl Linoleate-LDL by Fibroblast Monolayers. On day 7 of cell growth, the medium containing 5% LPDS was replaced with either 1 or 2 ml of fresh medium containing 5% LPDS and the indicated concentration of [^{3}H]CL-LDL added in a volume of 2–60 μl. Where indicated, 100 μM chloroquine diphosphate (Sigma) was added as either 10 or 20 μl of a 10 mM stock solution made up in Eagle's minimum essential medium adjusted to pH 7 with NaOH. After incubation at 37° in a humidified CO_2 incubator for the indicated time, each monolayer was washed twice at 4° with 3 ml of solution containing 0.15 M NaCl; 50 mM Tris·HCl, pH 7.4; and 2 mg/ml of bovine serum albumin, followed by a third wash with 3 ml of solution containing 0.15 M NaCl and 50 mM Tris·HCl, pH 7.4. The cells were then scraped into 1 ml of the latter buffer and centrifuged (900 $\times$ *g*, 10 min, 4°). The cell pellet was resuspended in 0.2 ml of H_2O, and a 10-μl aliquot was removed for protein determination (13). To the remaining suspension was added 4 ml of chloroform/methanol (2:1) containing 30 μg of [*4*-^{14}C]cholesterol (500 cpm). The mixture was agitated for 30 sec and allowed to stand at 24° for at least 30 min. To separate the phases, 0.8 ml of H_2O was added, and each tube was centrifuged (900 $\times$ *g*, 10 min, 4°). The lower phase was evaporated to dryness under air and the residue was resuspended in 0.1 ml of benzene/hexane (2:1) and spotted on silica gel sheets. Each sheet was developed one-third of its length with benzene/ethyl acetate (2:1), then dried in room air, and redeveloped to its full length in petroleum ether/benzene (2:1). The free cholesterol spot was cut out and radioactivity was determined in 10 ml of 0.5% 2,5-diphenyloxazole and 10% methanol in toluene. For each sample, a correction was made for the recovery of the internal [^{14}C]cholesterol standard, which averaged 84%. In all experiments, each value represents the mean of duplicate incubations. All results are expressed as pmol or nmol of [^{3}H]cholesterol formed from [^{3}H]CL-LDL per hr/mg of cell protein.

Hydrolysis of [^{3}H]Cholesteryl Linoleate-LDL by Cell-Free Fibroblast Extracts. On day 7 of cell growth after 24 hr in 5% LPDS, cells from 30 dishes were scraped into 30 ml of chilled buffer containing 0.15 M NaCl and 50 mM Tris·HCl, pH 7.4. After centrifugation (900 $\times$ *g*, 3 min, 4°), the pooled cell pellet was resuspended in 5 ml of the same buffer, washed once more in the same manner, and the final pellet suspended in 1 ml of H_2O. The extract was sonicated using a Bronson Sonifier with a microprobe and then centrifuged in a Beckman Microfuge (12,000 rpm, 30 sec, 24°); the resulting supernatant was used for enzyme assays. Each assay contained the following in a final volume of 50 μl: 0.1 M sodium acetate (pH 3 to 6.5) or 0.1 M Tris·HCl (pH 7–9) at the indicated pH; 0.5 mg/ml of bovine serum albumin; 5 mM 2-mercaptoethanol; 1 mM EDTA; the indicated concentration of [^{3}H]CL-LDL; and 34–96 μg of extract protein. After incubation at 37° for the indicated time, each reaction was stopped with 1 ml of chloroform/methanol (2:1) containing 30 μg of [^{14}C]cholesterol (500 cpm). The free [^{3}H]cholesterol was isolated and quantified as in the above monolayer assay.

RESULTS

Incubation of [^{3}H]cholesteryl linoleate with LDL in the presence of 10% dimethylsulfoxide was found empirically to yield a preparation in which the radioactive cholesteryl linoleate behaved as though it were an integral part of the LDL molecule, as indicated by the following observations. First, the [^{3}H]CL-LDL and LDL labeled with ^{125}I in its protein moiety were precipitated in parallel by an antibody to native LDL (Fig. 1). Second, the [^{3}H]CL-LDL retained full biologic activity, as shown by its ability to suppress 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and to activate cholesteryl ester formation in normal fibroblasts at the same concentrations as native LDL (data not shown). Third, hydrolysis of the [^{3}H]cholesteryl linoleate in LDL by intact fibroblasts was facilitated by the binding of the LDL to its specific cell surface receptor (see below).

When the [^{3}H]CL-LDL prepared by the dimethylsulfoxide method was incubated with monolayers of normal fibroblasts, the [^{3}H]cholesteryl linoleate was hydrolyzed, and the free [^{3}H]cholesterol that was formed accumulated within the cells (Fig. 2). The hydrolysis and accumulation of [^{3}H]cholesterol was linear with time up to at least 6 hr. The rate of hydrolysis was greater in normal cells than in cells from a

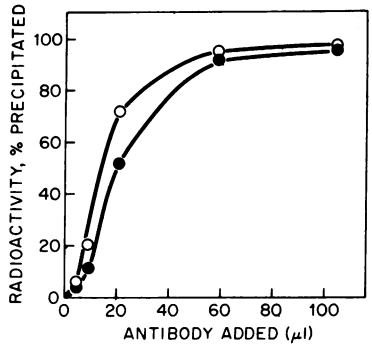

FIG. 1. Precipitation of ^{125}I-labeled LDL and [^{3}H]cholesteryl linoleate bound to LDL by incubation with anti-LDL antibody. To tubes containing either ^{125}I-labeled LDL (5 μg, 61,378 cpm) or [^{3}H]CL-LDL (5 μg, 70,000 cpm) was added each of the indicated amounts of a monospecific goat antibody to human LDL (Hyland) in a final volume of 300 μl containing 15 mM NaCl and 10 mM sodium phosphate, pH 7.4. Each reaction mixture was incubated for 2 hr at 37° followed by incubation for 16 hr at 4°. The immunoprecipitates were removed by centrifugation and washed twice with the above buffer. Radioactivity was determined either directly (^{125}I) or after dissolution in 100 μl of NCS (^{3}H). ●, ^{125}I-labeled LDL; ○, [^{3}H]CL-LDL.

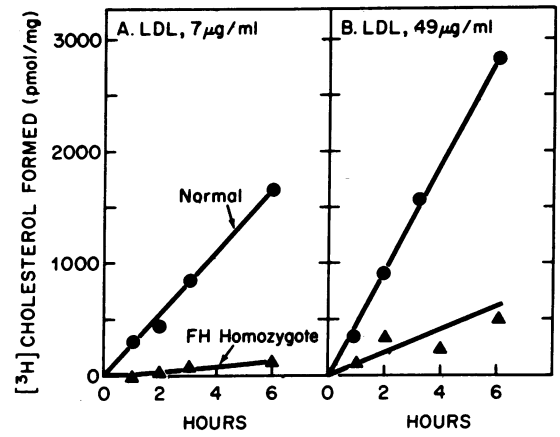

FIG. 2. Time course of hydrolysis of [^{3}H]cholesteryl linoleate bound to LDL by normal (●) and FH homozygote (▲) fibroblast monolayers. Cell monolayers were incubated under standard conditions with 2 ml of growth medium containing 5% LPDS and [^{3}H]CL-LDL (6671 cpm/nmol of cholesteryl linoleate) at an LDL concentration of either 7 μg of protein per ml (A) or 49 μg of protein per ml (B). At the indicated time, the cellular content of free [^{3}H]cholesterol was determined as described in *Methods*.

FH homozygote, suggesting that binding of the lipoprotein to its cell surface receptor facilitated the hydrolysis of its esterified cholesterol.

Further evidence for involvement of the LDL receptor in hydrolysis of the [^{3}H]CL-LDL is shown by the saturation kinetics in Fig. 3. As the concentration of [^{3}H]CL-LDL was increased in the medium surrounding normal fibroblasts, the rate of cholesteryl ester hydrolysis increased in a hyperbolic manner (Fig. 3A) similar to that previously observed for the saturable binding of ^{125}I-labeled LDL to its high affinity receptor (2). Moreover, the half-maximal rate for LDL hydrolysis was achieved at an LDL concentration similar to that giving half-maximal LDL binding (10–20 μg of protein per ml) (2). The FH homozygote cells, which lack functional LDL receptors, showed no evidence for a high affinity, saturable hydrolytic process (Fig. 3B). However, these mutant cells did show a low rate of cholesteryl ester hydrolysis that increased linearly with increasing LDL concentrations.

Chloroquine, a known inhibitor of lysosomal degradative processes in cultured cells (14), blocked the hydrolysis of LDL-bound [^{3}H]cholesteryl linoleate (Fig. 3). This agent has also been shown to inhibit completely the proteolytic hydrolysis of ^{125}I-labeled LDL in human fibroblasts without inhibiting binding or uptake of the lipoprotein(*).

The rate of hydrolysis of [^{3}H]CL-LDL in the absence of chloroquine was similar in cells from four normal subjects of different ages and sexes, and in each case chloroquine reduced hydrolytic activity by an average of 50-fold (Table 1). In the fibroblasts from four receptor-negative FH homozygotes, the rate of cholesteryl linoleate hydrolysis in the absence of chloroquine was severely reduced. The small amount of ^{3}H radioactivity found in the cholesterol spot in both normal and mutant cells in the presence of chloroquine may represent either residual hydrolytic activity occurring in the presence of the inhibitor or, more likely, a small contamination of the [^{3}H]CL-LDL with [^{3}H]cholesterol.

In the current experiments, the culture medium was discarded and only the cellular content of free [^{3}H]cholesterol was routinely quantified. This approach was validated by preliminary experiments showing that no detectable free

Table 1. Hydrolysis of [^{3}H]cholesteryl linoleate bound to LDL in monolayers of fibroblasts from normal subjects and subjects with the receptor-negative form of homozygous familial hypercholesterolemia

Subject	Age (yr)	Sex	Site of skin biopsy	No. of cell generations	[^{3}H]Cholesterol formed (pmol·hr^{-1}·mg^{-1}) (−) Chloroquine	(+) Chloroquine
Normal					(a)	(b)
(D.S.)	Newborn	M	Foreskin	18	194	2.9
(G.B.)	2	M	Abdomen	7	158	4.5
(M.Mc.)	5	F	Inquinal	7	165	4.0
(M.Z.)	34	M	Deltoid	20	211	2.9
				Mean	182	3.6
FH homozygote						
(M.C.)	6	F	Deltoid	18	12.5	8.8
(D.R.)	6	F	Deltoid	18	2.4	2.0
(L.L.)	10	M	Deltoid	13	9.8	2.5
(J.P.)	12	F	Abdomen	8	15.3	11.7
				Mean	10.0	6.3

Cell monolayers were incubated under standard conditions with 1 ml of growth medium containing 5% LPDS, 5.1 μg of protein per ml of [^{3}H]-CL-LDL (93,100 cpm/nmol of cholesteryl linoleate), and either no chloroquine or 100 μM chloroquine. After incubation for 6 hr the cellular content of free [^{3}H]cholesterol was determined as described in *Methods*.

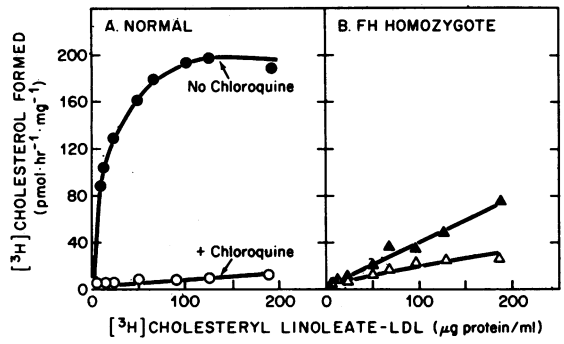

FIG. 3. Effect of LDL concentration on the rate of hydrolysis of [^{3}H]cholesteryl linoleate bound to LDL by normal (A) and FH homozygote (B) fibroblast monolayers incubated in the absence and presence of chloroquine. Cell monolayers were incubated under standard conditions with 2 ml of growth medium containing 5% LPDS, the indicated concentration of [^{3}H]CL-LDL (32,830 cpm/nmol of cholesteryl linoleate), and either no chloroquine (●,▲) or 100 μM chloroquine (○,△). After incubation for 6 hr, the cellular content of free [^{3}H]cholesterol was determined as described in *Methods*.

[^{3}H]cholesterol was released from the cells into the medium during the first 8 hr of these incubations.

The relation in normal fibroblasts between the metabolism of the cholesteryl ester and the protein components of LDL is shown by the time courses in Fig. 4. As previously reported, the amount of ^{125}I-labeled LDL bound to the cells reached a steady state between 2 and 3 hr (Fig. 4A), reflecting equal rates of cellular uptake and hydrolysis of LDL-protein (2). Hydrolysis of the [^{3}H]cholesteryl linoleate of LDL paralleled hydrolysis of the protein component (Fig. 4B). Inasmuch as the amount of LDL that contains 1 ng of protein also contains about 0.56 pmol of cholesteryl linoleate, it can be calculated that all of the [^{3}H]cholesteryl linoleate contained in each particle of LDL was hydrolyzed at the same time that the apoprotein component of the lipoprotein was hydrolyzed. A similar stoichiometric relation was observed when [^{3}H]cholesteryl oleate or [^{3}H]cholesteryl stearate was incorporated into LDL and incubated with normal fibroblasts.

To measure the reesterification of the free [^{3}H]cholesterol liberated from the hydrolysis of [^{3}H]CL-LDL, cells were incubated with [^{3}H]CL-LDL and an excess of [^{14}C]oleate (Fig. 4C). The total rate of formation of cholesteryl oleate, as monitored by [^{14}C]oleate incorporation, was much greater than could be accounted for by the incorporation of [^{3}H]cholesterol derived from the hydrolysis of [^{3}H]CL-LDL, indicating that unlabeled sterol was also available for esterification. Assuming that all of the cholesteryl esters in LDL were hydrolyzed at the same rate as cholesteryl linoleate and assuming that the unlabeled free cholesterol in LDL was also available to enter the cells, then for each molecule of [^{3}H]cholesterol derived from hydrolysis of [^{3}H]CL-LDL, two molecules of unlabeled cholesterol were also derived from LDL. It can be calculated from the data in Fig. 4B and C that at the end of 12 hr and for each 1 mg of cell protein, a total of 6600 pmol of cholesterol were derived from LDL (2200 pmol of [^{3}H]cholesterol and 4400 pmol of unlabeled cholesterol) and accumulated intracellularly. About 750 pmol (250 pmol of [^{3}H]cholesterol and 500 pmol of unlabeled cholesterol) were reesterified with oleate. However, since the [^{14}C]oleate curve indicates that at 12 hr 3200 pmol of cholesteryl [^{14}C]oleate were formed, the incoming cholesterol derived from LDL must have entered a pool of endogenous free cholesterol that supplied substrate for cellular esterification. If the LDL-derived cholesterol had equilibrated with the entire cellular pool of free cholesterol (which consists of about 1×10^5 pmol/mg of cell protein), the dilution of the incoming cholesterol would have been about 15-fold ($1 \times 10^5 \div 6600$) rather than the calculated value of 4.3-fold ($3200 \div 750$). Thus, it appears that cholesterol derived from LDL enters a cellular pool that is available for esterification and that this pool comprises about one-third of the total cellular content of free cholesterol.

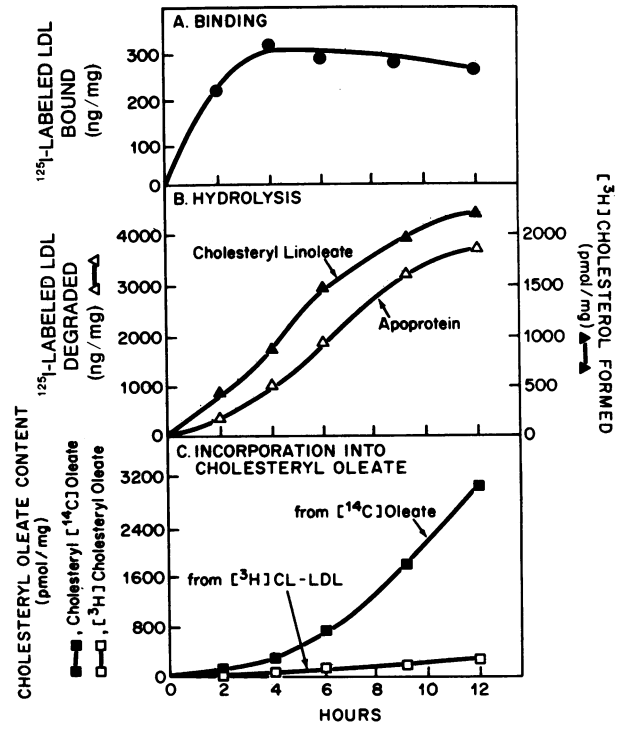

FIG. 4. Time course of LDL metabolism in normal fibroblast monolayers. One group of cells was incubated with 10 μg of protein per ml of ^{125}I-labeled LDL (340 cpm/ng of protein) and the amount of binding (●) and proteolytic hydrolysis (△) of the lipoprotein were determined as described (2). A second group of dishes was incubated with 10 μg of protein per ml of [^{3}H]CL-LDL (83,350 cpm/nmol of cholesteryl linoleate) and the amount of free [^{3}H]cholesterol formed (▲) was measured as described in *Methods*. A third group of dishes was incubated with 10 μg of protein per ml of [^{3}H]CL-LDL (83,350 cpm/nmol of cholesteryl linoleate) and 0.1 mM [*1*-^{14}C]oleate-albumin (19,550 cpm/nmol) (6) and the incorporation of [^{14}C]oleate (■) and [^{3}H]cholesterol (□) into cholesteryl oleate was determined by thin-layer chromatography on 5% silver nitrate/silica gel developed with benzene so as to separate cholesteryl oleate from cholesteryl linoleate. All three groups of dishes were incubated simultaneously under identical conditions. All incubations were conducted in 2 ml of growth medium containing 5% LPDS and a final concentration of 0.1 mM oleate-albumin, either labeled (Exp. C) or unlabeled (Exps. A and B). Each value represents the average of duplicate incubations.

Cell-free extracts of normal fibroblasts were capable of hydrolyzing [^{3}H]CL-LDL to free [^{3}H]cholesterol. The reaction showed a sharp optimum at pH 4, and no detectable [^{3}H]cholesterol was formed at pH 7 or above (Fig. 5). Hydrolysis of the [^{3}H]CL-LDL was linear with time for at least 3 hr, was directly proportional to the concentration of LDL up to about 300 μg of protein per ml (Fig. 6), and was eliminated by boiling the fibroblast extract (Fig. 6). At all concentrations of LDL, the rate of hydrolysis *in vitro* was at least 10- to 20-fold greater than the observed rate in intact cells. In contrast to the marked difference between intact normal and FH homozygote cells (Fig. 3), cell-free extracts from

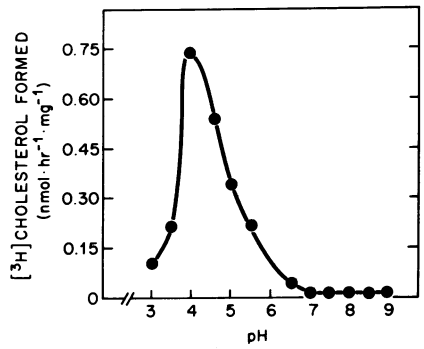

FIG. 5. Hydrolysis of [^{3}H]cholesteryl linoleate bound to LDL by cell-free extracts of normal fibroblasts at varying pH. [^{3}H]CL-LDL (0.42 μg of protein, 176,900 cpm/nmol of cholesteryl linoleate) was incubated with 96 μg of cell extract in a final volume of 50 μl for 2 hr at 37° under standard assay conditions at the indicated pH. The amount of free [^{3}H]cholesterol formed was determined as described in *Methods*. In a parallel set of incubations, no detectable [^{3}H]cholesterol was formed in the absence of cell extract at any pH.

both cell strains were equally effective in forming free [^{3}H]cholesterol from [^{3}H]CL-LDL at acid pH (Fig. 6).

DISCUSSION

The present studies demonstrate that hydrolysis of the cholesteryl esters in LDL proceeds in parallel with hydrolysis of the protein component of the lipoprotein. Like the previously demonstrated proteolytic reaction (2), hydrolysis of cholesteryl esters was markedly facilitated by the binding and cellular uptake of LDL, a process that is mediated through the LDL receptor. The essential role of the cell surface LDL receptor was emphasized by the finding that hydrolysis of cholesteryl linoleate in LDL was markedly reduced in intact cells from subjects with the homozygous form of FH.

As with proteolytic degradation of LDL*, the hydrolysis of the cholesteryl esters in LDL was inhibited by chloroquine and could be demonstrated to occur in cell-free extracts with a pH optimum of 4.0. In studies to be reported elsewhere, we have also shown that mutant cells from a subject with cholesteryl ester storage disease, which are severely deficient in a lysosomal acid lipase (15, 16), manifest a marked reduction in the ability to hydrolyze [^{3}H]CL-LDL despite normal binding and proteolytic hydrolysis of the lipoprotein. These observations are all consistent with a lysosomal localization for the hydrolysis of the cholesteryl esters in LDL.

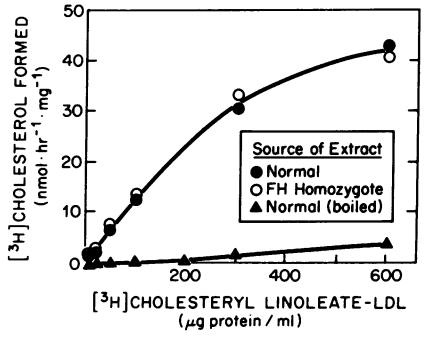

FIG. 6. Hydrolysis of [^{3}H]cholesteryl linoleate bound to LDL by cell-free extracts from normal and FH homozygote fibroblasts as a function of the concentration of LDL. The indicated concentration of [^{3}H]CL-LDL (52,262 cpm/nmol of cholesteryl linoleate) was incubated for 1 hr with the indicated extract [normal, either untreated (●) or boiled for 5 min (▲), 57 μg of protein; FH homozygote (○), 34 μg of protein] in a final volume of 50 μl at pH 4.3 under standard assay conditions. The amount of free [^{3}H]cholesterol formed was determined as described in *Methods*.

Considered in light of current concepts of macromolecular uptake in cultured cells (14, 17), our studies to date (1–8, *) lead us to formulate the following model for LDL metabolism in normal human fibroblasts. LDL binds to its cell surface receptor → the surface-bound LDL is incorporated into endocytotic vesicles → the interiorized vesicles containing bound LDL fuse with lysosomes → the cholesteryl ester and protein components of LDL are hydrolyzed by lysosomal enzymes → the liberated free cholesterol is transferred to cell membranes → cellular cholesteryl ester formation is stimulated and cellular cholesterol synthesis is suppressed by reciprocal changes in the activity of the two membrane-bound enzymes, fatty acyl-CoA:cholesteryl acyltransferase (cholesteryl ester formation) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (cholesterol synthesis).

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