Role of Microtubules in Low Density Lipoprotein Processing by Cultured Cells

RICHARD E. OSTLUND, JR., BARBARA PFLEGER, and GUSTAV SCHONFELD,
Lipid Research Center and Metabolism Division, Departments of
Preventive Medicine and Medicine, Washington University School
of Medicine, St. Louis, Missouri 63110

A B S T R A C T   The effect of the microtubule inhibitor colchicine on the metabolism of ^{125}I-low density lipoprotein (LDL) by cultured human skin fibroblasts and aortic medial cells was studied in vitro. Colchicine did not alter the binding of LDL to cell surface receptors. However, the rate of LDL endocytosis was reduced to 58% of that expected. Despite diminished endocytosis, LDL was found to accumulate within the cells to 165% of that expected, whereas the release of LDL protein degradation products into the medium was reduced to 34% of control, findings consistent with a reduced rate of intracellular LDL breakdown. Colchicine did not alter cell content of the acid protease which degrades LDL, nor did [^{3}H]colchicine accumulate in lysosomal fractions. However, colchicine did alter the intracellular distribution of both fibroblast lysosomes and endosomes. After colchicine, lysosomes tended to accumulate in the perinuclear region, whereas endosomes were found at the cell periphery. These findings are consistent with the hypothesis that ingested LDL is less available to lysosomal enzymes in the presence of colchicine. The actions of colchicine appear to be a result of destruction of cell microtubules. Lumicolchicine, a mixture of colchicine isomers which (unlike the parent compound) does not bind to the subunit of microtubules, was without effect.

The uptake and degradation of LDL by cultured cells consists of both a receptor-specific component and non-specific pinocytosis. Important differences must exist between these processes because even large amounts of LDL taken up and degraded by the nonspecific route fail to regulate key aspects of intracellular cholesterol metabolism. Colchicine selectively inhibited receptor-mediated LDL degradation. No effect was demonstrable on the nonspecific degradation of LDL by familial hypercholesterolemia fibroblasts grown in medium containing serum and added sterols. The degradation of bovine albumin by normal cells was also unaffected. Colchicine sensitivity appears to be a biochemical marker for the LDL receptor-specific metabolic pathway.

Cytochalasins inhibit crosslinking and polymerization of cell microfilaments (although other important cell effects also occur). Cytochalasin D reduced LDL degradation to 44% of that expected. This result and the actions of colchicine suggest that cytoskeletal components such as microtubules and possibly microfilaments facilitate normal LDL metabolism.

INTRODUCTION

Low density lipoprotein (LDL)[1] uptake and processing by cultured skin fibroblasts include several steps (1). LDL binds to cell surface receptors and is internalized in small endocytic vesicles that fuse with lysosomes (2). LDL is then degraded into free cholesterol and apoprotein fragments and the latter are released into the medium. There is reason to believe that cell microtubules and microfilaments might be involved in these processes.

Microtubules are 25-nm-diam, hollow core structures composed principally of tubulin (3). They appear to be required for saltatory movement of fibroblast lysosomes (4) and may also regulate the fusion of lysosomes with endocytic vacuoles (6–8) and alter the mobility of plasma membrane receptors (9). Tubulin is the only major cell protein that specifically binds colchicine (3, 5). The binding of colchicine causes depolymerization of microtubules, making colchicine useful as a probe for microtubular function in cells. Endocytosis is inhibited by colchicine in several systems (10, 11).

Microfilaments are morphologically quite distinct from microtubules. Composed principally of actin, they are 5–8 nm in diameter and may occur either in long

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[1] Abbreviations used in this paper: BSA, bovine serum albumin; LDL, low density lipoprotein.

bundles or in reticular networks (12). Cytochalasins interfere with the filament crosslinking necessary to form cytoplasmic microfilament gels (13) and also disrupt microfilaments directly at high concentration (14). The cytochalasins have other effects, however, including inhibition of glucose transport (15). Microfilaments are thought to affect a variety of endocytic (10) and secretory processes (16).

Because the interaction of LDL with cells involves endocytosis, we have studied the influence of colchicine and cytochalasins on LDL metabolism in cultured human skin fibroblasts and in aortic medial cells. We found that receptor-specific degradation of LDL was inhibited by colchicine, whereas nonspecific LDL degradation was not affected. Cytochalasin D also inhibited LDL degradation. Thus, "cytoskeletal" structures may importantly affect LDL metabolism.

METHODS

Reagents. Colchicine, cytochalasins B and D, chloroquine, crystallized bovine serum albumin (BSA), grade II sodium heparin, cholesterol, and acridine orange grade II were supplied by Sigma Chemical Co., St. Louis, Mo. 25-OH-cholesterol was purchased from Research Plus Laboratories, Denville, N. J. Fetal bovine serum was purchased from Gibco, Inc., Grand Island, N. Y. [³H]Colchicine was purchased from Amersham Corp., Arlington Heights, Ill. Lumicolchicine was prepared by the method of Mizel and Wilson (17). 50 μM colchicine in absolute ethanol was irradiated for 20 h in an open 1,000-ml glass beaker placed in a Baker VB-400 hood (Baker Co., Inc., Sanford, Maine) equipped with an overhead germicidal lamp. The ethanol was evaporated and residual colchicine was extracted into water three times. The dried lumicolchicine had a ratio of absorbances at 350/267 nm of 0.067 and a molar extinction coefficient at 267 nm in ethanol of 22,800. Lumicolchicine did not inhibit the binding of [³H]colchicine to brain tubulin when added in 50-fold excess.

Lipoproteins. LDL of density 1.019–1.063 was prepared from normal donors, iodinated by the iodine monochloride technique to a specific activity of 228±21 (SEM) cpm/ng and diluted with native LDL to give a specific activity of ≅100 cpm/ng LDL protein (18). All LDL additions were made as microgram per milliliter of LDL protein determined by the method of Sata et al. (19). BSA was iodinated by the method of Hunter and Greenwood (20) and purified by chromatography over Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N. J.).

Cells. Normal fibroblasts were derived from skin explants taken from a 3-mo-old male, a 33-yr-old male, and a 40-yr-old female. All normal lines gave similar results. Skin fibroblasts were also obtained from three patients with homozygous familial hypercholesterolemia. One is a 2-yr-old male who was classified as LDL "receptor-defective" on the basis of an LDL-induced:25-hydroxycholesterol-induced [¹⁴C]oleate incorporation ratio of 0.05:1, determined by published methods (21). Cells from two LDL "receptor-negative" patients, a 12-yr-old female (GM 996) and a 13-yr-old female (GM 1915) were purchased from the Human Genetic Mutant Cell Repository, Camden, N. J. Aortic cells were derived from an explant of thoracic aortic media taken at autopsy from a 2½-mo-old male who died of bronchopneumonia (26). Cell culture was as previously described (18). Fibroblasts were seeded at

75,000 per 35-mm-diam culture dish (or an equivalent amount for 60–100-mm dishes) and grown in 2 ml Eagle's Minimum Essential Medium (Gibco) containing 50 μg/ml streptomycin, 50 U/ml penicillin, 1.25 μg/ml amphotericin B (Fungizone, Squibb Corp., New York), and 15% fetal bovine serum. After 72 h the medium was replaced and the cells were grown for another 72 h. The medium then was removed, the cells were washed with 1 ml Puck's saline G (22), and 1 ml Eagle's Medium containing antibiotics, and 2.5 mg/ml human lipoprotein-deficient serum (18) was added. The cells were incubated 24 or 48 h before beginning an experiment. Aortic medial cells were grown similarly but in Dulbecco-modified Eagle's medium (Gibco) containing antibiotics, 15% fetal bovine serum, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids.

Cell ¹²⁵I-LDL accumulation and degradation (18). Cells were incubated at least 24 h in 5% lipoprotein-deficient human serum. The medium was removed and each 35-mm dish received 1 ml of fresh medium containing 5–500 μg/ml ¹²⁵I-LDL. The cells were incubated at 37°C for 1–17 h after which the experiment was terminated by removal of the medium and placement of the cells at 4°C. The cells were washed four times with 50 mM Tris-Cl, pH 7.4, in 0.15 M NaCl containing 2 mg/ml bovine albumin, and then twice with the preceding buffer without albumin. The cells were then dissolved in 0.1 N NaOH for determination of accumulated ¹²⁵I-LDL. In some experiments the cell surface ¹²⁵I-LDL was removed before dissolving the cells in NaOH by incubation for 1 h at 4°C with 1 ml Puck's saline G containing 10 mg/ml heparin with pH corrected to 7.4 (23). The saline G buffer was then aspirated and both it and cells, dissolved in 0.1 N NaOH, were counted separately. The noniodide ¹²⁵I-LDL degradation products soluble in 10% trichloroacetic acid (TCA) were determined in the removed culture medium (18). 1 ml medium was added to 0.25 ml 50% TCA. After 30 min at 4°C the tubes were centrifuged at 1,000 g for 10 min and 1.0 ml of the supernate was removed and treated with 10 μl 40% KI and 40 μl 30% H_2O_2 for 5 min at room temperature. 2 ml $CHCl_3$ was added and the tubes were thoroughly vortexed after which 0.5 ml of the aqueous phase was counted. Blank dishes containing culture medium and ¹²⁵I-LDL, but no cells, were routinely employed. Noniodide TCA soluble counts observed in blank dishes were subtracted from those of experimental dishes unless otherwise stated. Cell protein was determined by the method of Lowry et al. (24). Statistical comparisons were performed by Student's t test. Means±SE of the mean are presented.

¹²⁵I-LDL degradation by cell sonicates (25). Five 100-mm dishes of normal fibroblasts were grown in the usual fashion including 24-h incubation in medium containing 2.5 mg/ml lipoprotein-deficient serum. The cells were washed and scraped into 15 ml 50-mM Tris-Cl, pH 7.4, containing 0.15 M NaCl. The cells were collected at 90 g for 3 min, washed in 5 ml of the same buffer, suspended in 0.75 ml water, and sonicated for 1.5 min on ice. Experiments were performed by incubating 20 μl of cell sonicate with 10 μl ¹²⁵I-LDL containing ≅100,000 cpm (final concentration, 5 μg/ml) in 100 μl 0.1 M sodium acetate, pH 4.0, containing 5 mM dithiothreitol and 1 mM EDTA for 1 h at 37°C. ¹²⁵I-LDL degradation products were then determined as described above.

Estimation of microtubules. An assay for microtubules in tissue culture cells is presented in detail elsewhere (36). Fibroblasts were taken to a 37°C room and washed six times with 0.15 M NaCl. A 100-mm dish of cells was washed once (with 1 ml) and homogenized in 0.3 ml microtubule stabilization buffer consisting of 10 mM sodium phosphate (pH 6.95), 0.5 mM $MgCl_2$, 0.5 mM guanosine 5'-triphosphate, 0.5 mM EGTA, 50% glycerol, and 5% dimethylsulfoxide, and placed

at 4°C. Microtubules were separated from free tubulin by sedimentation of the former at 130,000 g for 10 min in a Beckman Airfuge (Beckman Instruments, Inc., Fullerton, Calif.). The microtubule-containing pellet was rehomogenized in 0.3 ml depolymerization buffer consisting of 0.25 M sucrose, 0.5 mM GTP, and 10 mM sodium phosphate, pH 6.95. After incubation at 4°C for 1 h, the solution containing depolymerized microtubule subunits was clarified by centrifugation at 130,000 g for 10 min. Colchicine binding activity of the supernate was determined by incubation at 37°C for 1 h with 1 μM [^{3}H]colchicine. The binding of [^{3}H]colchicine to microtubule protein fractions under these conditions is proportional to the amount of tubulin present. Microtubules in the absence of colchicine treatment contained 36±2% of the total cell tubulin.

RESULTS

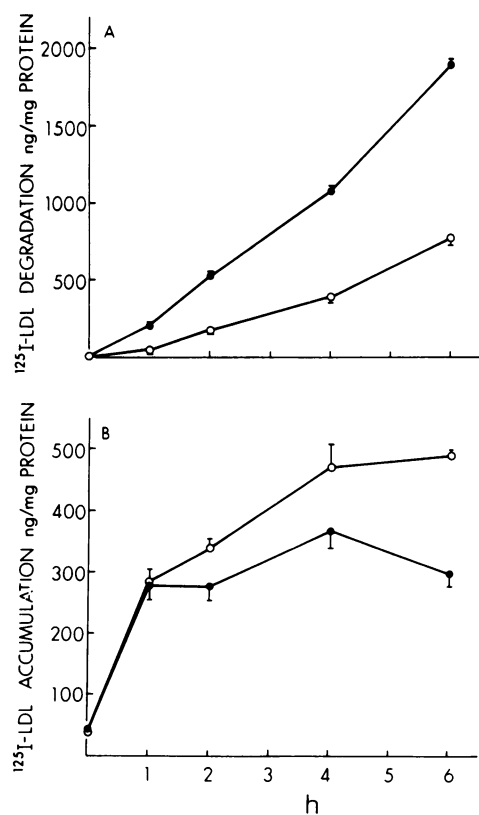*Colchicine and LDL degradation.* ^{125}I-LDL metabolism was studied in human skin fibroblasts that

FIGURE 1 Effect of colchicine on ^{125}I-LDL degradation and accumulation. Triplicate dishes of normal fibroblasts were prepared for LDL binding with a 24-h incubation in lipoprotein-deficient serum (Methods). Some dishes received colchicine in a small volume of water sufficient to achieve a final concentration of 10 μM 2 h before the experiment began. At zero time the medium was removed and replaced with fresh medium containing 10 μg/ml ^{125}I-LDL either with or without 10 μM colchicine. The cells were incubated for varying times at 37°C. ^{125}I-LDL degradation (panel A) and accumulation (panel B) are presented; ●, control; ○, 10 μM colchicine. Bars indicate SEM.

had been previously treated with lipoprotein-deficient serum for 24 h to stimulate LDL uptake. ^{125}I-LDL was added to control dishes and to dishes that had been preincubated for 2 h with 10 μM colchicine. Cellular degradation and accumulation of ^{125}I-LDL were then observed in the presence and absence of colchicine for up to 6 h (Fig. 1). Colchicine significantly reduced ^{125}I-LDL degradation at all time points, the average reduction being to 33.9±3.8% of the control value (Fig. 1A; $P < 0.01$). The accumulation of ^{125}I-LDL by colchicine-treated cells was increased after the 1st h and at 6 h was 165±2.1% of control (Fig. 1B; $P < 0.01$). In both control and colchicine-treated cells >94% of the accumulated radioactivity was precipitable in 15% TCA at 0°C.

Colchicine had similar effects in human aortic medial smooth muscle cells (Table I). At 10 μM it reduced ^{125}I-LDL degradation to 39% of control. A similar degree of inhibition of ^{125}I-LDL degradation was noted in the presence of a 50-fold excess of unlabeled LDL. Accumulation of LDL was not significantly affected by colchicine.

A dose-response curve for the inhibition of ^{125}I-LDL degradation in fibroblasts by colchicine is found in Fig. 2. Normal fibroblasts were preincubated for 2 h with colchicine and also received colchicine during a 2-h incubation period with ^{125}I-LDL. The lowest concentration of colchicine at which a significant reduction in LDL degradation occurred was 1 μM where 61±5.0% of the expected release of LDL degradation products occurred. But the addition of up to 100 μM colchicine caused no further decline in the release of degradation products. Lumicolchicine, a mixture of photochemical colchicine isomers which does not bind tubulin, had no significant effect upon ^{125}I-LDL degra-

TABLE I
Effect of Colchicine on ^{125}I-LDL Metabolism in Human Aortic Medial Cells

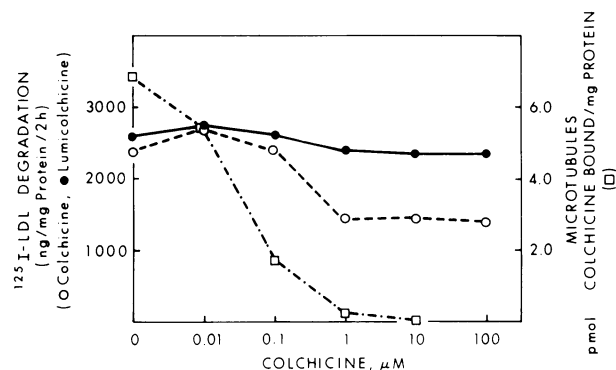|	^{125}I-LDL	
	Degradation	Accumulation
	ng/mg protein/2 h	
Control	39.0±1.6	73.6±6.9
10 μM Colchicine	15.2±1.6*	60.5±2.3
500 μg/ml Unlabeled LDL	16.1±1.4*	21.3±1.0*

Triplicate 35-mm dishes were plated with 100,000 cells, grown as described in Methods, and prepared with lipoprotein-deficient serum. Colchicine-treated dishes received the drug during a 2-h initial incubation period. At time zero, the medium was replaced with fresh medium containing 10 μg/ml ^{125}I-LDL and either colchicine, unlabeled LDL, or no addition, and the cells were incubated 2 h at 37°C.
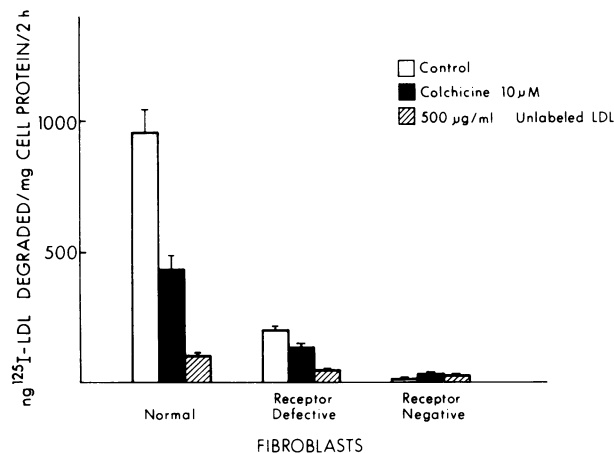* $P < 0.01$ compared to control.

dation at concentrations from 0.01 μM to 100 μM (Fig. 2).

The effect of colchicine on fibroblast microtubule content was also studied (Fig. 2). Microtubules were reduced to 25% of expected levels by 0.1 μM colchicine and to 4% by 1 μM colchicine. Significant effects of colchicine on ^{125}I-LDL degradation were noted at 1 μM and greater concentrations. Thus, the degradation of ^{125}I-LDL was reduced only in the absence of significant numbers of microtubules. However, a significant proportion of ^{125}I-LDL was degraded even in the absence of detectable microtubules at 10 μM colchicine (Fig. 1).

Effect of colchicine on nonspecific degradation. Fibroblasts from a normal subject and from patients with "receptor-defective" and "receptor-negative" hypercholesterolemia were tested for degradation of ^{125}I-LDL in a single assay (Fig. 3). Colchicine treatment (10 μM) reduced ^{125}I-LDL degradation to 45% of that expected in normal cells, to 68% of that expected in LDL "receptor-defective" cells, and had no effect on "receptor-negative" cells. Because little LDL was degraded by cells deficient in receptors during a 2-h period, other experiments were performed (Table II). Colchicine reduced ^{125}I-LDL degradation to 52% of control during a 6-h incubation using normal fibroblasts in which LDL receptors had been induced by preincubation for 24-h in lipoprotein-deficient serum.

FIGURE 3 Effect of colchicine on ^{125}I-LDL metabolism in fibroblasts of differing phenotype. Fibroblasts from a normal subject and from patients with "receptor-defective" and "receptor-negative" familial hypercholesterolemia were prepared for LDL binding by incubation 24 h in lipoprotein-deficient serum. Colchicine was added in a small volume of water to certain dishes at 10 μM final concentration 2 h before the experiment began. At zero time the medium was replaced with fresh medium containing 50 μg/ml ^{125}I-LDL and either 500 μg/ml unlabeled LDL, 10 μM colchicine, or no addition. The dishes were incubated for 2 h at 37°C. ^{125}I-LDL degradation was assessed as described in the Methods section. Results are mean ± 1 SEM.

FIGURE 2 Effect of colchicine on microtubules and ^{125}I-LDL degradation. Dishes used for the evaluation of ^{125}I-LDL degradation were pretreated for 2 h with concentrated stock solutions of aqueous colchicine or lumicolchicine in ethanol, whereas untreated dishes received either 0.2% water or 0.2% ethanol. At time zero the medium was replaced with fresh medium containing the same additives as well as 100 μg/ml ^{125}I-LDL and the dishes were incubated for an additional 2 h before determination of ^{125}I-LDL degradation. Dishes used for quantitation of microtubules (Methods) received colchicine or water for 3 h without a medium change. ^{125}I-LDL degradation in the presence or absence of colchicine and cell microtubule content were not altered by the amount of ethanol employed. Apparent degradation of ^{125}I-LDL in dishes without cells was not subtracted from the values plotted. Each point represents the mean of three dishes.

Colchicine had no significant effect on ^{125}I-LDL degradation in normal fibroblasts in which receptors had been suppressed by incubation in fetal bovine serum as well as cholesterol and 25-OH-cholesterol. In these experiments the concentration of ^{125}I-LDL was raised to 500 μg/ml to increase the relative amount of nonspecific LDL degradation. LDL receptor-negative cells were also prepared by incubation in fetal bovine serum and added sterols. No effect of colchicine on ^{125}I-LDL degradation was seen at either high (250 μg/ml) or low (5 μg/ml) concentrations of ^{125}I-LDL. Thus, the effect of colchicine was observed only in cells having receptors for LDL.

The action of colchicine on the degradation of ^{125}I-BSA was investigated in a 17-h incubation (Table II). Despite a 42% reduction in the degradation ^{125}I-LDL in parallel dishes, no effect of colchicine was observed on ^{125}I-BSA degradation.

Cytochalasin and LDL degradation. The effect of cytochalasins on LDL degradation is shown in Table III. Cytochalasin D at 10 μM reduced ^{125}I-LDL degradation to 44% of that expected. Similar to colchicine, the inhibition of degradation was not complete and even 100 μM cytochalasin D was no more effective than 1 μM. Cytochalasin B had no effect on LDL degradation at 10 μM (Table III).

Mechanism of colchicine action. A number of experiments was performed to localize the site of

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TABLE II

Effect of Colchicine on Nonspecific LDL Degradation

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Cells, preincubation media, and doses of tracer	[125]I Tracer degradation			
	6-h Incubation		17-h Incubation	
	−Colchicine	+Colchicine (10 μM)	−Colchicine	+Colchicine (10 μM)
	ng/mg/6 h		*ng/mg/17 h*	
Normal cells preincubated in LPDS,* 500 μg/ml ^{125}I-LDL	7,636±86	3,943±165‡		
Normal cells preincubated in 25-OH-cholesterol + cholesterol, 500 μg/ml ^{125}I-LDL	798±130	562±56		
Receptor-negative cells preincubated in 25-OH-cholesterol + cholesterol, 5 μg/ml ^{125}I-LDL	27.2±2.0	31.3±6.4		
Receptor-negative cells preincubated in 25-OH-cholesterol + cholesterol, 250 μg/ml ^{125}I-LDL	1,378±50	1,224±130		
Normal cells preincubated in LPDS, 5 μg/ml ^{125}I-LDL			3,960±159	2,301±110‡
Normal cells preincubated in LPDS, 24 μg/ml ^{125}I-bovine albumin			106±5	97±8

Three to five dishes per condition were preincubated for 24 h in either 5% lipoprotein-deficient serum (to induce LDL receptors) or in 15% fetal bovine serum containing 2 μg/ml 25-OH-cholesterol and 25 μg/ml cholesterol (to prevent induction of receptors). Dishes treated with colchicine received the drug in a small volume of water 2 h before as well as during the experiment. At time zero the medium of all dishes was replaced with Eagle's Medium + 5% lipoprotein-deficient serum containing tracer and the indicated drugs. Cells were incubated at 37°C for 6 or 17 h. The amount of apparent tracer degradation in dishes containing no cells was subtracted from all entries.
* LPDS = Eagle's Medium + 5% lipoprotein-deficient human serum.
‡ P < 0.001 with respect to no colchicine.

colchicine action in fibroblasts. Receptor-bound ^{125}I-LDL that had not yet been internalized was eluted into a solution of heparin, which is known to bind LDL (23) (Table IV). Colchicine at 10 μM had no significant effect upon heparin-releasable LDL.

The rate of LDL internalization was next considered. This was estimated as the sum of ^{125}I-LDL accumulation and degradation in Fig. 1. At all time points, the rate of LDL uptake was significantly less in colchicine-treated cells, varying from 70% of that expected at 1 h to 58% at 6 h (P < 0.05). Hence, colchicine decreased the rate of LDL endocytosis. But at the same time the accumulation of ^{125}I-LDL in colchicine-treated cells was always equal to or greater than that of controls (Fig. 1). Because the heparin-elutable portion was unaffected by colchicine, this suggested that the rate of intracellular ^{125}I-LDL processing must have been decreased to an even greater extent than the rate of endocytosis.

The possibility that colchicine inhibited the activity of enzyme(s) degrading LDL was tested. Colchicine inhibited ^{125}I-LDL protein degradation in cell sonicates at very large concentrations (Table V). An inhibition of only 22% occurred at 100 μM colchicine, whereas 1 μM was normally effective in our cell culture system (Fig. 2). However, it was possible that colchicine might have accumulated in lysosomes to sufficient concentration for inhibition of LDL degradation to occur during

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TABLE III

Effect of Cytochalasins on ^{125}I-LDL Degradation

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	^{125}I-LDL degradation*
	ng/mg protein
2-h Preincubation	
Control	1,626±47
Dimethylsulfoxide, 0.05 (vol/vol)	1,504±64
Cytochalasin D, 10 μM	663±31‡
Cytochalasin B, 10 μM	1,736±52
30-min Preincubation*	
Control	2,380±138
Cytochalasin D, 0.1 μM	2,089±97
Cytochalasin D, 1 μM	1,327±102‡
Cytochalasin D, 10 μM	1,263±30‡
Cytochalasin D, 0.1 mM	1,295±62‡

Normal fibroblasts were prepared for LDL binding by incubation in lipoprotein-deficient serum. The triplicate dishes were preincubated with the appropriate drug in 0.05% dimethylsulfoxide (final concentration) for either 2 h or 30 min. At time zero the medium was replaced with fresh medium containing drug and 100 μg/ml ^{125}I-LDL. The dishes were then incubated 2 h at 37°C and degradation of ^{125}I-LDL was determined.
* Apparent degradation of ^{125}I-LDL in dishes without cells was not subtracted from experimental results shown.
‡ P < 0.05 compared to control or dimethylsulfoxide-treated dishes.

TABLE IV
Heparin-Releasable ^{125}I-LDL Binding

	^{125}I-LDL		
	Cell accumulation		
	Heparin elutable	Heparin resistant	Degradation
	ng/mg protein		
Control	61.9±6.0	506±32	1,183±73
+ 10 µM Colchicine	60.8±9.3	850±145	452±50*
+ 500 µg/ml Unlabeled LDL	2.6±0.7*	25±6*	36±6*

Fibroblasts prepared for LDL binding were incubated 2 h at 37°C with fresh medium containing 5 µg/ml ^{125}I-LDL after which binding and degradation assays were performed as described in Methods. Cells treated with colchicine received the drug for a 2-h preincubation period as well as during the 2-h incubation.
* P < 0.01 compared to control.

the incubation of the intact cells. To test this possibility [3H]colchicine (1 µM) was incubated with cells for 3 h and then the cells were washed and fractionated (Table VI). Over 97% of the colchicine counts were found in the 130,000 g supernate, and very little colchicine was found in any particulate fractions. Thus, concentration of colchicine in lysosomes did not occur, confirming predictions based on its pK of 12.5 (27). Therefore, direct inhibition of lysosomal enzymes could not have been a significant mechanism of colchicine action. Nor did colchicine affect the cell content of lysosomal LDL degrading enzymes. Fibroblasts were treated with 10 µM colchicine or a similar small amount of water for 4 h and then sonicated in water to release lysosomal enzymes. ^{125}I-LDL degradation in the sonicates was 4,171±330 ng/mg protein per h in con-

TABLE V
Effect of Colchicine on In Vitro Degradation of ^{125}I-LDL

	LDL degradation	
Inhibitor concentration	Colchicine	Chloroquine
mM	% of control±SEM	
0	100±2.3	
0.1	78.4±2.6	78.3±7.3
1.0	49.5±7.7	54.6±10.6
10	18.7±2.7	18.5±1.3
70	4.1±1.5	3.8±0.7

The degradation of ^{125}I-LDL to TCA-soluble noniodide products by a fibroblast sonicate was determined at pH 4 as described in Methods. Each number represents the mean of three determinations. Basal ^{125}I-LDL degradation was 17.2-20.2 ng/h.

TABLE VI
Fractionation of Fibroblasts after Exposure to [3H]Colchicine

	Fraction	Protein fraction	cpm
	cpm	µg	µg
Homogenate	14,493	266	54.5
500 g pellet	119	58	2.1
130,000 g pellet	405	45	9.0
130,000 g supernate	14,114	97	146

Normal fibroblasts were prepared as if for LDL binding by incubation in 5% lipoprotein-deficient serum for 24 h. [3H]Colchicine at a final concentration of 1.0 µM, 536,900 cpm/ml, was added in a small volume and the cells were incubated at 37°C for 3 h. They were then washed six times with 0.15 M NaCl and homogenized 100 strokes with a Teflon-glass (DuPont Co., Wilmington, Del.) homogenizer in 0.3 ml 0.25 M sucrose containing 1 mM EDTA pH 7.0 and centrifuged at 500 g for 10 min. The pellet was washed with the same buffer and recentrifuged. The supernate was centrifuged at 130,000 g (average) for 10 min in a Beckman Airfuge. The pellets were dissolved in 100 µl 1 N NaOH for counting. Numbers are the mean of two experiments.

trols and 4,103±228 ng/mg protein per h in colchicine-treated cells.

Chloroquine, too, inhibited LDL degradation in cell sonicates at high concentration (Table V). However, fibroblast lysosomes concentrate chloroquine to over 25 mM under conditions similar to ours (28). The experiment of Table VII gave further evidence that the

TABLE VII
Effects of Colchicine and Chloroquine on ^{125}I-LDL Metabolism

Condition	^{125}I-LDL accumulation	^{125}I-LDL degradation	Degradation + accumulation
	ng/mg protein/4 h		
Control	370±28	1,087±50	1,457±55
Colchicine, 10 µM	474±36	398±36*	872±72*
Chloroquine, 60 µM	796±39*	46±11*	843±27*
Colchicine, 10 µM + chloroquine, 60 µM	402±23‡	6.7±4.8‡	410±24‡

Normal fibroblasts were grown and prepared for LDL binding as described in Methods. A small amount of colchicine stock was added to the medium of all colchicine-treated dishes 2 h before the experiment. At time zero the medium was aspirated and 10 µg/ml ^{125}I-LDL in fresh medium containing drugs or no addition was added. The cells were incubated at 37°C for 4 h. Each number represents the mean of three or four plates.
* P < 0.001 compared to control.
‡ P < 0.05 compared to chloroquine alone.

mechanism of action of chloroquine differed from that of colchicine. Cells were incubated with chloroquine alone, colchicine alone, or both drugs. Chloroquine increased ^{125}I-LDL accumulation in cells to 215% of that expected. Colchicine also increased ^{125}I-LDL accumulation slightly (but in this case not significantly). But when the drugs were added simultaneously, antagonism resulted such that the increased LDL accumulation seen with chloroquine was significantly reduced to control levels. In contrast, the effect of the two drugs to lower ^{125}I-LDL degradation was additive. This experiment suggested that colchicine increased fibroblast ^{125}I-LDL accumulation by a different mechanism than chloroquine. The effect of colchicine occurred before lysosomal hydrolysis, the presumed site of chloroquine action.

Because colchicine has been reported to alter the intracellular spatial orientation of cell organelles (5), the distribution pattern of lysosomes and LDL-containing endosomes was studied with and without colchicine treatment (Fig. 4). Lysosomes visualized by supravital acridine orange staining (37, 38) were normally diffuse in cytoplasm, but tended to accumulate in the perinuclear region after colchicine (Fig. 4A, B). LDL-containing endosomes, also diffuse in the cytoplasm of untreated cells, assumed a distinctly peripheral pattern after colchicine treatment when visualized by indirect immunofluorescence (41) (Fig. 4C, D). Lumicolchicine 10 μM had no effect on the distribution of either lysosomes or endosomes (results not shown).

DISCUSSION

Colchicine inhibited ^{125}I-LDL degradation by cultured human fibroblasts and aortic medial cells to 35–55% of that expected. The effect appears to be a result of disaggregation of microtubules (Fig. 2). An amount of colchicine (1 μM) that resulted in disaggregation of 96% of the microtubules also inhibited ^{125}I-LDL degradation. However, there was no effect on LDL degradation at 0.1 μM where microtubule disaggregation was 75% complete, suggesting that inhibition of ^{125}I-LDL degradation required near total destruction of the microtubular cytoskeleton. Lumicolchicine (17), a mixture of colchicine isomers that does not bind to the subunit protein of microtubules, had no effect on ^{125}I-LDL degradation.

The pathway of LDL degradation by tissue culture cells involves several discrete steps (1). LDL binds to a plasma membrane receptor which undergoes endocytosis in a vesicle. Fusion with lysosomes occurs after which LDL is hydrolyzed to free cholesterol and protein fragments in the secondary lysosome. We have studied the effect of colchicine on these processes.

The binding of ^{125}I-LDL to its surface receptor was determined by heparin elution of cell-associated counts (23). Colchicine had no effect on receptor-bound LDL (Table IV). However, the rate of endocytosis, calculated as the sum of ^{125}I-LDL cell accumulation and formation of ^{125}I-protein degradation products, was reduced to 60% of that expected by colchicine (Table VII, Fig. 1).

Despite a reduced rate of endocytosis, colchicine increased cell accumulation of ^{125}I-LDL (Fig. 1B). This required that colchicine reduce the effective rate of degradation of internalized ^{125}I-LDL even more, either by reducing the fusion of endosomes with lysosomes or by directly inhibiting the activities or decreasing the amounts of the degrading enzymes.

There was no alteration in cell content of the lysosomal enzyme activity degrading LDL protein. Likewise, colchicine did not accumulate in lysosomes in sufficient amount to inhibit the degradative enzymes directly (Tables V and VI). The presence of adequate activities of degradative enzymes and of increased amounts of LDL in the cells suggested that colchicine produced a defect in endosome-lysosome fusion. This was supported by the altered distribution patterns of lysosomes stained with acridine orange (5, 37, 38) and LDL endosomes visualized by immunofluorescence (Fig. 4). After colchicine treatment, lysosomes were found more prominently around the nucleus, whereas LDL endosomes were located more peripherally near the plasma membrane. Colchicine has been shown to inhibit endosome-lysosome fusion in several other cell types (6–8) and to stop the long saltatory movements of lysosomes in fibroblast-like cells (4).

Degradation of ^{125}I-LDL by cultured fibroblasts can occur after LDL uptake either by specific cell receptors or by nonspecific pinocytosis (1). The LDL receptors greatly increase LDL uptake and degradation at low LDL concentrations (5–10 μg/ml), and this results in regulation of intracellular cholesterol metabolism as manifested by increased esterification of cholesterol, decreased hydroxymethylglutaryl coenzyme A reductase activity, and fewer LDL receptors (1, 31). However, at high LDL concentrations (e.g., 200 μg/ml) the uptake and degradation of LDL by nonspecific pinocytosis may equal that mediated by receptors (18, 31). But when LDL is taken up and degraded even in large amount by the nonspecific route, there is no regulation of cholesterol metabolism (31). The biochemical basis of the difference between the specific and nonspecific "uptake pathways" is not known.

We have found that colchicine inhibited only specific receptor-mediated ^{125}I-LDL degradation (Table II, Fig. 3). Colchicine had no effect on degradation of ^{125}I-LDL taken up by nonspecific pinocytosis in receptor-negative cells treated with sterols, or in normal cells treated with sterols and assayed with a large concentration of ^{125}I-LDL (500 μg/ml) to increase the relative amount of nonspecific uptake (Table II). Colchicine had no effect
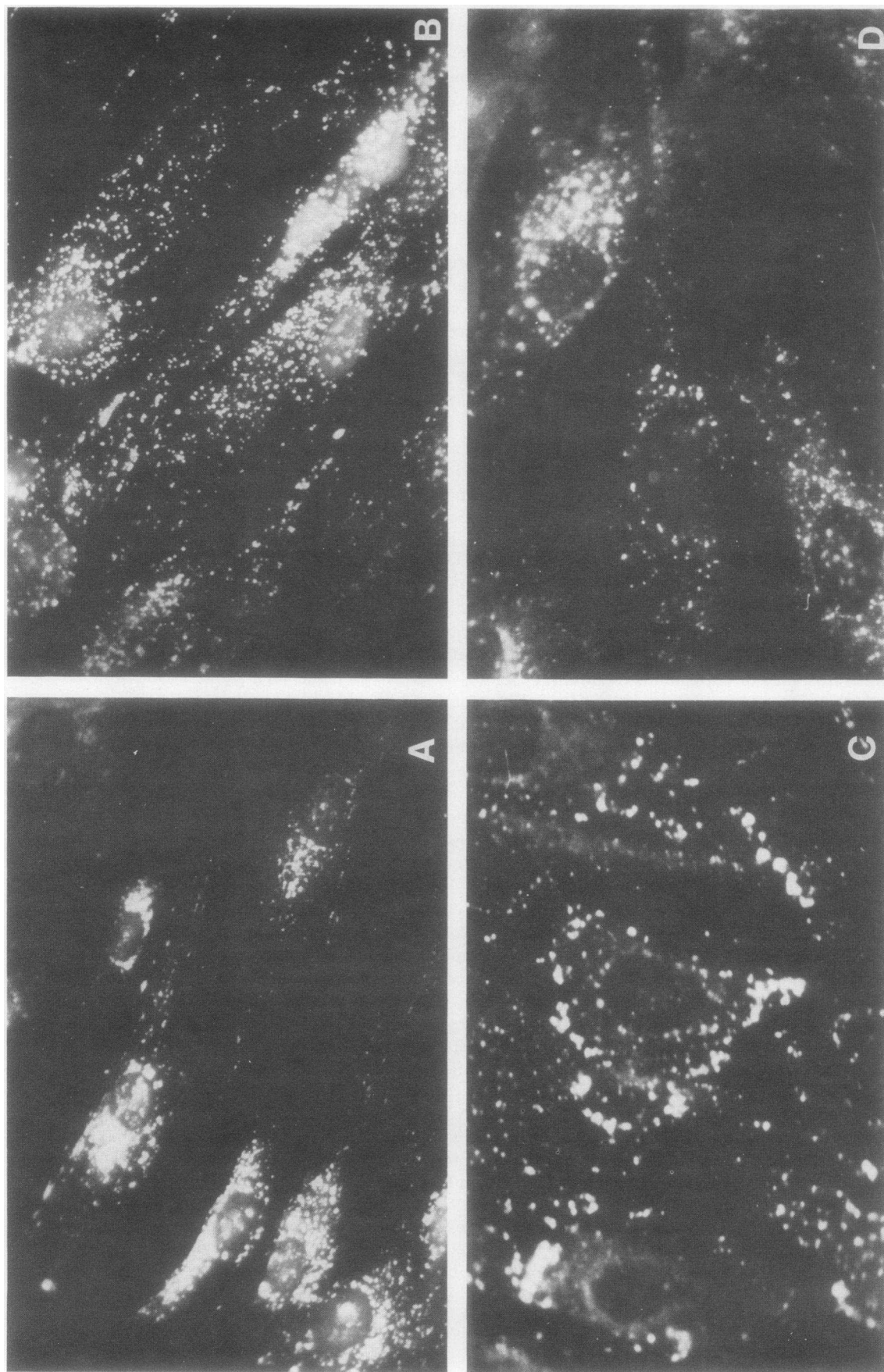
FIGURE 4 Effect of drugs on lysosomes and on intracellular localization of LDL. Normal fibroblasts were grown and prepared as if for LDL binding in 35-mm dishes containing a glass coverslip. Cells were pretreated for 2 h with 10 μM colchicine (A, C), no addition (B, D), and stained with acridine orange (A, B), or anti-LDL antisera (C, D). For acridine orange staining the medium was removed and cells were treated with 0.13 mM acridine orange in 0.15 M NaCl containing 15 mM Hepes, pH 7.4, for 7 min at room temperature. The cells were then washed quickly with the dye-free buffer, the medium was replaced, and the cells were incubated at 37°C for 1 h. Coverslips were inverted onto glass slides and observed in a Leitz fluorescence microscope with filters selected for fluorescein. For immunolocalization of LDL, 100 μg/ml of LDL was added in Eagle's Medium followed by incubation for 2 h at 37°C. The medium was removed and the cells were washed, air dried, treated at −20°C with methanol for 4 min and acetone for 2 min, and then immunostained with rabbit anti-human LDL and fluorescinated goat anti-rabbit gamma globulin as described (41).

on the degradation of ^{125}I-BSA by normal cells under conditions where a significant reduction in ^{125}I-LDL degradation was noted (Table II). Thus, colchicine sensitivity appears to be a biochemical marker for the receptor-specific pathway. This is consistent with published data demonstrating that uptake of small particles by pinocytosis is not sensitive to colchicine (32).

The selective inhibitory effect of colchicine on receptor-specific LDL degradation could have several explanations. Receptor-LDL binding may serve to organize microtubules to assist in the uptake and degradation of the bound material. The binding of concanavalin A to the plasma membrane has resulted in the apparent polymerization, or at least redistribution, of microtubules to greater density near the binding site (34, 35). Two types of endocytic vesicles have been identified in human skin fibroblasts (2); coated vesicles that contain the specific LDL receptors, and morphologically distinct, smaller, uncoated vesicles that do not contain LDL receptors but that are capable of nonspecific uptake of fluid from the cell exterior. It is possible that the preferential effect of colchicine on receptor-specific ^{125}I-LDL degradation is because of an effect on the intracellular movement of the coated, but not the uncoated, vesicles. It is also possible that receptor-specific degradation of LDL occurs in specialized lysosomes and nonspecific degradation occurs in other lysosomes. For example, patients with Chediak-Higashi syndrome demonstrate normal fusion of phosphatase-containing lysosomes with endocytic vesicles, but fusion of peroxidase and B-glucuronidase-containing lysosomes is reduced (33). This suggests that fusion of some, but not all, lysosomes with endosomes may be facilitated by microtubules. The possibility that nonspecific degradation might occur in the cytoplasm must also be kept in mind (39), although published evidence indicates that nonspecific LDL degradation by familial hypercholesterolemia fibroblasts is highly chloroquine-sensitive and hence presumably occurs in lysosomes (40).

Cytochalasin D, but not cytochalasin B, also inhibited ^{125}I-LDL degradation (Table III). Cytochalasins have several effects upon cells (13–15). However, the effect of cytochalasin D on LDL degradation could be a result of structural disorganization of actin-containing microfilaments, and the hypothesis that microfilaments are involved in cell LDL processing deserves further study.

We believe that agents active against microtubules and microfilaments may be useful probes for further defining the LDL receptor pathway.

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REFERENCES

1. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* **46:** 897–930.
2. Anderson, R. G. W., M. S. Brown, and J. L. Goldstein. 1977. Role of coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell.* **10:** 351–364.
3. Olmstead, J. B., and G. G. Borisy. 1973. Microtubules. *Annu. Rev. Biochem.* **42:** 507–540.
4. Freed, J. J., and M. M. Lebowitz. 1970. The association of a class of saltatory movements with microtubules in cultured cells. *J. Cell Biol.* **45:** 334–354.
5. Ostlund, R., and I. Pastan. 1975. Fibroblast tubulin. *Biochemistry.* **14:** 4064–4068.
6. Malawista, S. E., and P. T. Bodel. 1967. The dissociation by colchicine of phagocytosis from increased oxygen consumption in human leukocytes. *J. Clin. Invest.* **46:** 786–796.
7. Zurier, R. B., S. Hoffstein, and G. Weissman. 1973. Cytochalasin B: effect on lysosomal enzyme release from human leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* **70:** 844–848.
8. Oronsky, A., L. Ignarro, and R. Pepper. 1973. Release of cartilage mucopolysaccharide-degrading neutral protease from human leukocytes. *J. Exp. Med.* **138:** 461–472.
9. Oliver, J. M., and R. B. Zurier. 1976. Correction of the characteristic abnormalities of microtubule function and granule morphology in Chediak-Higashi syndrome with cholinergic agonists. Studies in vitro in man and in vivo in the beige mouse. *J. Clin. Invest.* **57:** 1239–1247.
10. Allison, A. C., and P. Davies. 1974. Interaction of membranes, microfilaments, and microtubules in endocytosis and exocytosis. *In* Advances in Cytopharmacology. B. Ceccarelli, F. Clementi, and J. Meldolesi, editors. Raven Press, New York. **2:** 237–247.
11. Wolff, J., and J. A. Williams. 1973. The role of microtubules and microfilaments in thyroid secretion. *Recent Prog. Horm. Res.* **29:** 229–285.
12. Ishikawa, H., R. Bischoff, and H. Holtzer. 1969. Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. *J. Cell Biol.* **43:** 312–328.
13. Hartwig, J. H., and T. P. Stossel. 1976. Interactions of actin, myosin, and an actin-binding protein of rabbit pulmonary macrophages. III. Effects of cytochalasin B. *J. Cell Biol.* **71:** 295–303.
14. Spudich, J., and S. Lin. 1972. Cytochalasin B, its interaction with actin and actomyosin from muscle. *Proc. Natl. Acad. Sci. U. S. A.* **69:** 442–446.
15. Mizel, S. B., and L. Wilson. 1972. Inhibition of the transport of several hexoses in mammalian cells by cytochalasin B. *J. Biol. Chem.* **247:** 4102–4105.
16. Ostlund, R. E., Jr. 1977. Contractile proteins and pancreatic beta-cell secretion. *Diabetes.* **26:** 245–254.
17. Mizel, S. B., and L. Wilson. 1972. Nucleoside transport in mammalian cells. Inhibition by colchicine. *Biochemistry.* **11:** 2573–2578.
18. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. *J. Biol. Chem.* **249:** 5153–5162.
19. Sata, T., R. J. Havel, and A. L. Jones. 1972. Characterization of subfractions of triglyceride-rich lipoproteins separated by gel chromatography from blood plasma of

normolipemic and hyperlipemic humans. *J. Lipid Res.* **13**: 757–768.

20. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.).* **194**: 495–496.

21. Goldstein, J. L., S. E. Dana, G. Y. Brunschede, and M. S. Brown. 1975. Genetic heterogeneity in familial hypercholesterolemia: evidence for two different mutations affecting functions of low-density lipoprotein receptor. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 1092–1096.

22. Puck, T. T., S. J. Cieciura, and A. Robinson. 1958. Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. *J. Exp. Med.* **108**: 945–956.

23. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low-density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. *Cell.* **7**: 85–95.

24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.

25. Goldstein, J. L., G. Y. Brunschede, and M. S. Brown. 1975. Inhibition of the proteolytic degradation of low-density lipoprotein in human fibroblasts by chloroquine, concanavalin A, and Triton W. R. 1339. *J. Biol. Chem.* **250**: 7854–7862.

26. Ross, R. 1971. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell Biol.* **50**: 172–186.

27. DeDuve, C., T. DeBarsy, B. Poole. A. Trouet, P. Tulkens, and F. Van Hoof. 1974. Commentary lysosomotropic agents. *Biochem. Pharmacol.* **23**: 2495–2531.

28. Wibo, M., and B. Poole. 1974. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B_1. *J. Cell Biol.* **63**: 430–440.

29. Pesanti, E. L., and S. G. Axline. 1975. Colchicine effects on lysosomal enzyme induction and intracellular degradation in the cultivated macrophage. *J. Exp. Med.* **141**: 1030–1046.

30. Robbins, E., and N. K. Gonatas. 1964. Histochemical and ultrastructural studies on hela cell cultures exposed to spindle inhibitors with special reference to the interphase cell. *J. Histochem. Cytochem.* **12**: 704–711.

31. Goldstein, J. L., and M. S. Brown. 1976. The LDL pathway in human fibroblasts: a receptor-mediated mechanism for the regulation of cholesterol metabolism. *Curr. Top. Cell Regul.* **11**: 147–181.

32. Casley-Smith, J. R. 1969. Endocytosis: the different energy requirements for the uptake of particles by small and large vesicles into peritoneal macrophages. *J. Microsc. (Oxf.).* **90**: 15–30.

33. Stossel, T. P., R. K. Root, and M. Vaughan. 1972. Phagocytosis in chronic granulomatosis disease and the Chediak-Higashi syndrome. *N. Engl. J. Med.* **286**: 120–123.

34. Hoffstein, S., R. Soberman, I. Goldstein, and G. Weissman. 1976. Concanavalin A induces microtubule assembly and specific granule discharge in human polymorphonuclear leukocytes. *J. Cell Biol.* **68**: 781–787.

35. Albertini, D. F., and E. Anderson. 1977. Microtubule and microfilament rearrangements during capping of concanavalin A receptors on cultured ovarian granulosa cells. *J. Cell Biol.* **73**: 111–127.

36. Pipeleers, D. G., M. A. Pipeleers-Marichal, P. Sherline, and D. M. Kipnis. 1977. A sensitive method for measuring polymerized and depolymerized forms of tubulin in tissues. *J. Cell Biol.* **74**: 341–350.

37. Robbins, E., P. I. Marcus, and N. K. Gonatas. 1964. Dynamics of acridine orange-cell interaction. II. Dye-induced ultrastructural changes in multivesicular bodies (acridine orange particles). *J. Cell Biol.* **21**: 49–62.

38. Robbins, E., and P. I. Marcus. 1963. Dynamics of acridine orange-cell interaction. I. Interrelationships of acridine orange particles and cytoplasmic reddening. *J. Cell Biol.* **18**: 237–250.

39. Etlinger, J. D., and A. L. Goldberg. 1977. A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 54–58.

40. Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 3178–3182.

41. Schonfeld, G., E. Bell, and D. H. Alpers. 1978. Intestinal apoproteins during fat absorption. *J. Clin. Invest.* **61**: 1539–1550.