Rapid intracellular transport of LDL-derived cholesterol to the plasma membrane in cultured fibroblasts

Dawn L. Brasaemle[1] and Alan D. Attie[2]

Departments of Biochemistry and Comparative Biosciences, University of Wisconsin-Madison, Madison, WI 53706

Abstract The kinetics of low density lipoprotein (LDL) cholesterol transport to the plasma membrane of Chinese hamster ovary (CHO) cells was studied. LDL was reconstituted with [^{3}H]cholesteryl linoleate and added to CHO cells in a pulse-chase experiment. The internalization and lysosomal cleavage of reconstituted LDL (rLDL) [^{3}H]cholesteryl linoleate to free [^{3}H]cholesterol occurred with a half-time of 37 min after a 30-min lag. The rate of transport of released [^{3}H]cholesterol to the plasma membrane was measured by brief (20–30 sec) cholesterol oxidase treatment of intact, adherent cells; the half-time of transport was 42 min. The similarity in the rate of free cholesterol release from rLDL and transport of this cholesterol to the plasma membrane suggests very rapid transport of rLDL cholesterol from the lysosome to the plasma membrane. Cells were shown to be intact throughout the cholesterol oxidase treatment by the absence of cell-derived lactate dehydrogenase (LDH) activity or K^+ in the assay buffer. —**Brasaemle, D. L., and A. D. Attie.** Rapid intracellular transport of LDL-derived cholesterol to the plasma membrane in cultured fibroblasts. *J. Lipid Res.* 1990. **31:** 103–112.

Supplementary key word cholesterol oxidase

Cholesterol is an integral component of mammalian cell membranes. Cells obtain cholesterol from two sources, endogenous synthesis and the uptake of plasma cholesterol. The primary carrier of plasma cholesterol in humans is low density lipoprotein (LDL), which delivers cholesterol to cells via receptor- and non-receptor-mediated pathways (1–4). Both biosynthetic and exogenously derived cholesterol are used by cells as structural components of membranes; excess cholesterol is stored in cytoplasmic cholesteryl ester droplets. The mechanisms by which cells transport and compartmentalize cholesterol are not well understood.

Cholesterol is unequally distributed among cell membranes. Membrane cholesterol distribution has been determined by subcellular fractionation (5–7); plasma membranes are relatively enriched in cholesterol (0.5–0.8 cholesterol/phospholipid molar ratio), while endoplasmic reticulum (0.1–0.3 cholesterol/phospholipid molar ratio) and mitrochondria (0–0.1 cholesterol/phospholipid molar ratio) have much lower cholesterol contents, with Golgi (0.4–0.5 cholesterol/phospholipid molar ratio) being intermediate between endoplasmic reticulum and plasma membrane. Electron microscopy studies visualizing filipin binding to cholesterol in membranes have demonstrated polarity of cholesterol distribution within a membrane subpopulation; the *trans* Golgi is relatively enriched with cholesterol, similar to the plasma membrane, while the *cis* Golgi cholesterol composition is more similar to that of the endoplasmic reticulum (8).

The mechanisms for maintaining the nonuniform distribution of cholesterol among cell membranes are unknown. The phospholipid distribution among cellular membranes is also unequal and may determine the affinity of a membrane for cholesterol (9–12). Although the phospholipid components of the various cellular organelles may influence cholesterol partitioning, the phospholipid content alone cannot account for the disparate subcellular cholesterol composition (12). Unidirectional transport of cholesterol from the endoplasmic reticulum to the plasma membrane may explain this intracellular cholesterol gradient (13–15).

Three mechanisms for intracellular lipid transport have been proposed (reviewed in ref. 16): *1*) aqueous diffusion of lipid monomers; *2*) vesicular transport; and *3*) lateral diffusion between contiguous membrane compartments.

Abbreviations: LDL, low density lipoprotein; CHO, Chinese hamster ovary; LDH, lactate dehydrogenase; FBS, fetal bovine serum; NCS, newborn calf serum; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; LDS, lipoprotein-deficient serum.

[1]Present address: Division of Nutritional Sciences, Savage Hall, Cornell University, Ithaca, NY 14853.

[2]To whom correspondence should be addressed at: Department of Biochemistry. 420 Henry Mall, University of Wisconsin-Madison, Madison, WI 53706-1569. (Reprints not available from the authors.)

Aqueous diffusion of cholesterol monomers has been shown between isolated membranes and synthetic lipid vesicles (17, 18) but is difficult to demonstrate between membranes of intact cells. Several intracellular sterol-binding proteins have been described and a role for these proteins as cholesterol carriers has been demonstrated in model systems (19, 20). Light microscopic studies have suggested vesicular transport of fluorescent phospholipids (16). Several subcellular fractionation studies have identified a vesicular compartment enriched in newly synthesized cholesterol (13, 21–24); however, the origin and function of this compartment have not been elucidated. Lateral diffusion of cholesterol between contiguous membrane compartments is the basis of a membrane flow hypothesis of cholesterol transport. This model is dependent on membrane bridges between cellular organelles. Although these interconnections have been visualized by microscopy, the structures may have been artifacts of tissue fixation (16).

This study estimates the rate of transport of cholesterol from LDL to the plasma membranes of CHO cells in culture. We have used the enzyme cholesterol oxidase as a tool to measure the rate of intracellular transport of lipoprotein cholesterol. Cholesterol oxidase converts cholesterol to cholestenone, and has been used to sample plasma membrane cholesterol in mammalian cells. We have defined conditions for the use of cholesterol oxidase in the study of the transport of radiolabeled cholesterol from reconstituted LDL (rLDL) to the plasma membrane, and have estimated the rate of this process.

EXPERIMENTAL PROCEDURES

Materials

[1,2-^{3}H(N)]cholesterol (53.0 Ci/mmol) was purchased from New England Nuclear. Radiochemical purity was determined by thin-layer chromatography (TLC) on silica gel G plates (Analtech, Inc.) developed in benzene–ethyl acetate 2:3. Radioactive bands were located by scanning plates with a Bioscan System 200 Imaging Scanner. Mevinolin was kindly donated by A. W. Alberts (Merck, Sharp & Dohme Research Laboratories, Rahway, NJ). Cholest-4-en-3-one was obtained from Steraloids, Inc. (Wilton, NH), linoleic anhydride was obtained from Nu-Chek Prep, Inc. (Elysian, MN), and stigmasta-4,22-dienone and 4-pyrrolidinopyridine were obtained from Sigma. Ham's F12 medium, penicillin (10,000 units/ml)/streptomycin (10,000 μg/ml) solution and trypsin/EDTA (lyophilized 20 ml 0.5% trypsin, 5.3 mM EDTA, 10X) were obtained from GIBCO Laboratories. Fetal bovine serum and newborn calf serum were obtained from HyClone Laboratories, Inc. Cholesterol oxidase (EC 1.1.3.6; *Brevibacterium* sp., 4.06 IU/mg) was obtained from Beckman Instruments. Solvents used in lipid extractions were redistilled reagent grade. Corning cell culture 75-cm^2 flasks and 100-mm dishes were used.

Cell culture

Chinese hamster ovary (CHO) cells (CHO-K1, American Type Culture Collection) were maintained in Ham's F12 medium supplemented with 5% fetal bovine serum (FBS)–newborn calf serum (NCS) (1:1), 1 mM glutamine, and penicillin (100 units/ml) and streptomycin (100 μg/ml) (P/S) (growth medium) in 75 cm^2 flasks. Cultures were maintained in a 37°C incubator in a 5% CO_2, humid atmosphere. Prior to experiments, cells were rinsed with sterile phosphate-buffered saline (PBS) (145 mM NaCl, 5 mM KCl, 5 mM sodium phosphate, pH 7.4) and released from dishes with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA). Cells were then plated into 100-mm dishes at a density of approximately 10^5 cells per dish and were used for experiments within 72 h (approximately 60–80% confluence).

Preparation of reconstituted low density lipoproteins

[^{3}H]cholesteryl linoleate was prepared by shaking [^{3}H]cholesterol with linoleic anhydride and 4-pyrrolidinopyridine in benzene under a nitrogen atmosphere in an amber vial for 16 h at 25°C (25). [^{3}H]Cholesteryl linoleate was purified by TLC on a silica gel G plate developed in hexane–diethyl ether–acetic acid 80:25:1. Radioactivity was located by scanning the plate with a Bioscan System 200 Imaging Scanner.

Human LDL was collected by preparative ultracentrifugation (ϱ = 1.019–1.063 g/ml) (26). LDL was reconstituted by the method of Krieger (27), and then dialyzed against PBS. Reconstituted LDL (rLDL) recovery was determined by measuring protein by the method of Lowry et al. (28), cholesterol by enzymatic assay (Sigma kit #351), and radioactivity of an aliquot of rLDL dissolved in BioSafe II scintillant (Research Products International Corp.). The ratio of cholesteryl linoleate to protein of rLDL used in the described experiments was 0.84–0.86.

Cholesterol oxidase assay for plasma membrane cholesterol

Adherent subconfluent CHO cells in 100-mm dishes were rinsed three times with PBS, then three times with assay buffer (310 mM sucrose, 1 mM $MgSO_4$, 0.5 mM sodium phosphate, pH 7.4) at 25°C. Assay buffer was added to the dishes followed by cholesterol oxidase (0.01 to 0.2 IU/ml final concentration, except where noted). The enzyme reaction was conducted at 25°C for 20 to 30 sec, except where noted. The reaction was stopped by removing the assay buffer containing the enzyme and immediately extracting the dishes with hexane–isopropanol 3:2 containing stigmasta-4,22-dienone as an internal standard for sterol extraction efficiency and quantitation. Lipid residues were stored at −20°C. The assay buffer

from the cell incubations was saved for subsequent determinations of cell permeability. Lactate dehydrogenase (LDH) assays were conducted immediately when applicable; samples for K^+ determinations were stored at −20°C.

Previously published cholesterol oxidase assay conditions

The cholesterol oxidase assay of Lange and Ramos (29) was used to compare the levels of cholesterol oxidation and cell permeability under those conditions to the same parameters under our conditions. Following the former methods, subconfluent CHO cells grown in 100-mm dishes were rinsed two times with PBS, then released wtih trypsin/EDTA for 5 min at 37°C, and transferred to 1.5 ml Eppendorf microfuge tubes. The released cells were pelleted by brief centrifugation (20 sec) in a Fisher Microfuge Model 235A (13,000 *g*) at 4°C. The cells were rinsed twice with PBS by resuspending and briefly centrifuging the cells at 4°C. The cells were then suspended in 1% glutaraldehyde in PBS and incubated for 15 min on ice, vortexing occasionally. The cells were rinsed three times with assay buffer (310 mM sucrose, 0.5 mM sodium phosphate, pH 7.4), and resuspended. The tubes were then incubated at 37°C for 15 min; 2 IU/ml cholesterol oxidase was added and the tubes were incubated for up to 45 min at 37°C. At various times during the incubation, cells were pelleted by brief centrifugation and the supernatants were removed for K^+ determinations. The pellets were then extracted as described above.

Measurement of transport of rLDL cholesterol to the plasma membrane

Cell growth conditions for cholesterol transport experiments. CHO cells were seeded into 100-mm dishes at approximately 10^5 cells/dish. Cells were grown for 24–48 h before the medium was changed to Ham's F12 medium supplemented with 2% lipoprotein-deficient serum (NCS $\varrho > 1.21$ g/ml fraction), 1 mM glutamine, and P/S (medium A). After 24 h, the medium was changed to Ham's F12 medium supplemented with 2% LDS, 1 mM glutamine, P/S, 2 μg/ml mevinolin to inhibit sterol synthesis, and 5 μg/ml mevalonate to provide precursor for nonsterol isoprenoid synthesis but not sterol synthesis (30) (medium B), and the cells were grown for an additional 24 h.

rLDL pulse-chase experiments. Dishes of subconfluent CHO cells were rinsed twice with PBS prewarmed to 37°C. During the rinses, the cells remained attached to the dishes, but assumed a spherical appearance. HEPES-buffered F12 supplemented with 5 mg/ml bovine serum albumin (BSA), 1 mM glutamine, P/S, 2 μg/ml mevinolin, and 5 μg/ml mevalonate (medium C) was added to the dishes, and the cells were incubated for 10–20 min at 37°C to allow the cells to fully reattach to the dishes. The cells were then incubated at 4°C for 30–40 min to allow temperature equilibration. The medium was changed to medium C containing rLDL (20 to 36 μg rLDL protein/ml and 7.5×10^5 to 2.8×10^6 cpm rLDL [^{3}H]cholesteryl linoleate/dish). The cells were incubated at 4°C for 2.5 h on a rotary shaker. The medium containing rLDL was removed and the cells were rinsed three times with PBS at 4°C. The medium was changed to either medium B (4 experiments), or medium B containing 40 μg/ml unlabeled LDL (two experiments). The cells were then incubated at 37°C. At various times, the medium was removed and saved for cholesterol efflux determinations and the cells were subjected to our modified cholesterol oxidase assay for plasma membrane cholesterol. An average of 35% oxidation of cellular free cholesterol was obained in six experiments. Determination of LDH activity or K^+ released into the assay buffer during cholesterol oxidase treatment was used to determine cell permeability.

Efflux measurements. Cellular efflux of [^{3}H]cholesterol into the chase medium of cells was determined in three experiments. Lipids were extracted from chase medium samples by the method of Bligh and Dyer (31) and the extracts were dried in a Savant Speed-Vac Concentrator. Radioactive sterol content of the lipid extracts was analyzed by TLC followed by scintillation counting. Mass of effluxed free cholesterol derived from rLDL was calculated from the starting specific radioactivity of rLDL [^{3}H]cholesteryl linoleate.

Data analysis. The rates of [^{3}H]cholesteryl linoleate cleavage and [^{3}H]cholesterol transport to the plasma membrane were calculated by first determining the plateau of maximum specific radioactivity for cholesterol and cholestenone using the HYPERO program (32), which fits the data to an equation for a hyperbola. The specific radioactivity values for each data point in the initial part of the curve were expressed as the percent of the maximum specific radioactivity, and half-times were calculated from plotted data.

Other methods

Lipid quantitation. Dried lipid extracts (from hexane–isopropanol extractions) contained excess white precipitate, hence were re-extracted by the method of Bligh and Dyer (31) to further purify the lipids. The chloroform phase was dried under nitrogen to yield a colorless residue. Lipids in radioactive samples (rLDL pulse-chase experiment samples) were analyzed by TLC and gas–liquid chromatography; lipids in nonradioactive samples (control experiments) were quantitated by gas–liquid chromatography only. For radioactive samples, the lipids were resolubilized in chloroform, and half of the sample was spotted onto silica gel G TLC plates beside nonradioactive cholesterol, cholestenone, and cholesteryl ester standards using a TLC multispotter. The plates were developed in hexane–diethyl ether–acetic acid 80:25:1.5. The standards were

visualized by iodine vapors and radioactivity was located by scanning the plates with a Bioscan System 200 Imaging Scanner. Radioactive bands were scraped into scintillation vials, Econofluor (NEN Research Products) was added, and the sample radioactivity was determined using a Packard 1900CA Tri-Carb liquid scintillation analyzer. The remainder of the sample was used for sterol mass quantitation by gas–liquid chromatography using a Varian 3400 Gas Chromatography with an online Hewlett-Packard 3390A integrator. The samples were concentrated under nitrogen, and one third of the remaining sample was injected into a 4-ft column of Dexsil 300 on 100/120 Supelcoport (Supelco) with nitrogen as carrier gas (30 cm^2/min) and a temperature program of 250°C for 8 min, followed by a 6°C/min increase to 360°C. The masses of cholesterol and cholestenone were determined by comparison of peak areas to known quantities of the internal standard, stigmasta-4,22-dienone.

Lactate dehydrogenase assays. LDH activity was determined (33) in assay buffer from cells as a measure of cell permeability during cholesterol oxidase treatments. Released LDH activity from cell incubations with or without enzyme was compared to the total amount of enzyme activity in cells solubilized by 0.5% sodium deoxycholate in double-distilled H_2O.

Measurement of K^+ release. The concentration of intracellular K^+ released by cells incubated with or without cholesterol oxidase was determined as a measure of cell permeability. The K^+ content of assay buffer removed from cell incubations was determined by atomic absorption spectroscopy. The A_{766} of assay buffer samples was determined on a Perkin-Elmer Model 403 Atomic Absorption spectrophotometer. The concentration of K^+ released from cholesterol oxidase-treated and control cells was compared to the total concentration of K^+ released from cells solubilized with 0.5% sodium deoxycholate in ddH_2O. Sodium deoxycholate failed to release K^+ from glutaraldehyde-fixed cells; hence, total cellular K^+ was determined relative to cholesterol levels in unfixed cells and the total cellular content of K^+ for glutaraldehyde-fixed cells was calculated from their cholesterol content.

RESULTS

Modification of the cholesterol oxidase assay for plasma membrane cholesterol

Cholesterol oxidase converts cholesterol to cholest-4-en-3-one. A previous study demonstrated that cholesterol oxidase induces cell lysis under some conditions (34); hence, we tested the integrity of CHO cells treated with cholesterol oxidase by measuring the release of intracellular potassium during enzyme treatment. We used the method of Lange and Ramos (29) to measure cholesterol oxidase-accessible cholesterol in Chinese hamster ovary (CHO) cells. CHO cells were released from dishes with trypsin/EDTA, rinsed, fixed with glutaraldehyde, and then treated with cholesterol oxidase for 45 min. The oxidation of cellular free cholesterol increased rapidly with time of enzyme treatment to 100% oxidation by 15 min (**Fig. 1**). The concentration of extracellular K^+ increased to 80% of total cell K^+, indicating concomitant permeabilization of cell membranes to cellular ions. Similar results were obtained for cells that were treated in the same manner without glutaraldehyde fixation; both cholesterol oxidation and the extracellular K^+ concentration increased with time of enzyme treatment to the same extent as in glutaraldehyde-fixed cells. No increase in released K^+ was measured for cells incubated in the absence of enzyme over 45 min.

We modified the cholesterol oxidase assay for plasma membrane cholesterol. In order to eliminate potentially injurious effects of trypsin treatment on cells and to reduce sample processing time, adherent cells were briefly treated with cholesterol oxidase (20 to 30 sec, except where noted) and immediately extracted while still on the dishes. Processing time to lipid extraction was less than 2 min after removal of the cells from 37°C incubation conditions, thus allowing rapid acquisition of multiple time

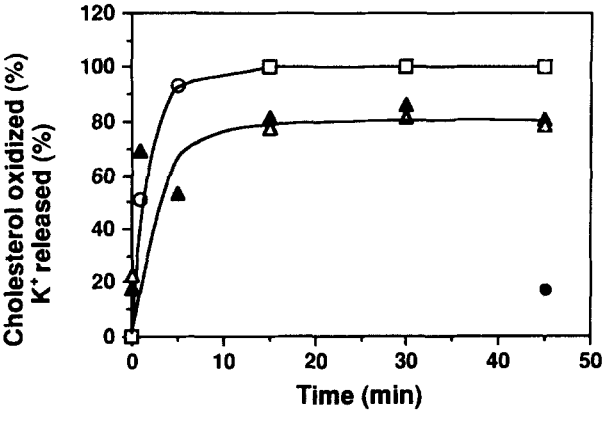


Fig. 1. Cholesterol oxidation and intracellular K^+ release in suspended cells treated with cholesterol oxidase with or without prior glutaraldehyde fixation. Cells were released from 100-mm dishes by trypsin treatment, rinsed with PBS at 4°C, and then fixed for 15 min with 1% glutaraldehyde in PBS at 4°C (fixed cells), or incubated for 15 min in PBS at 4°C (unfixed cells). The cells were washed in assay buffer, resuspended in this buffer, and then incubated for 15 min at 37°C. Cholesterol oxidase was added (2 IU/ml), and the cells were incubated at 37°C for the times indicated. At these times, assay buffer was removed from cells following brief centrifugation, and the K^+ content of the buffer was determined. The lipid content was determined, and the % cholesterol oxidation was determined from the mass of cholestenone/total mass of cholesterol + cholestenone. (○,□) Represent the % cholesterol oxidized of fixed and unfixed cells, respectively; (▲△) represent the % of total cellular K^+ relased from fixed and unfixed cells, repectively; (●) represents the % of total cellular K^+ released from control cells treated as described above without cholesterol oxidase, with or without glutaraldehyde fixation. The data shown are from one representative experiment out of three.

points and minimizing potential passive diffusion of cholesterol between membranes during sample processing.

We carried out this assay at various concentrations of cholesterol oxidase and found that the oxidation of cellular cholesterol increased with increasing concentrations of enzyme (**Fig. 2A and B**). To determine the permeability of cells during treatment with cholesterol oxidase, the release of a cytosolic enzyme, lactate dehydrogenase (LDH), and intracellular K^+ into the assay buffer were measured (Fig. 2A and B). Released LDH activity increased only slightly with increased cholesterol oxidation (Fig. 2A). Cells treated with 1 IU/ml cholesterol oxidase released 0 to 3% more of total cellular LDH activity than the no-enzyme controls in four separate experiments. Since LDH is a large molecule (M_r = 140 kD), and therefore may not be a sensitive indication of permeabilization of cells to cholesterol oxidase (M_r = 56 kD), we decided to use a more rigorous measure of cell permeability, the release of intracellular K^+. When less than 40% of cellular free cholesterol was oxidized by cholesterol oxidase, less than 10% of total cell K^+ was released (Fig. 2B). When more than 40% of cellular cholesterol was oxidized, the extent of cellular K^+ release more closely paralleled the percent of cholesterol oxidation.

Measurement of the rate of transport of LDL cholesterol to the plasma membrane in CHO cells using a radiolabeled tracer

LDL was reconstituted with [^{3}H]cholesteryl linoleate (27). CHO cells were preincubated under conditions that enhance the uptake of lipoprotein cholesterol by depleting cellular sterol levels (lipoprotein-deficient serum, LDS) and inhibit sterol synthesis (mevinolin). The cells were incubated with reconstituted LDL (rLDL) at 4°C to allow binding of the particles to cell surface receptors without internalization. The cells were then incubated in chase medium with or without excess cold LDL at 37°C to allow endocytosis of rLDL with subsequent transport to lysosomes for cleavage of the lipoprotein constituents. At various times, samples of cells were removed to determine incorporation of the cholesterol tracer into cellular free cholesterol and plasma membrane pools. Free [^{3}H]cholesterol from cleavage of rLDL [^{3}H]cholesteryl linoleate appeared in the cells following a 30-min lag (**Fig. 3A and B**); the specific radioactivity of cellular free cholesterol increased rapidly following the lag and approached a plateau by 2 h. Cholesterol oxidase was used to determine the rate of appearance of [^{3}H]cholesterol at the plasma membrane, indicated by the increase in specific radioactivity of cholestenone. The conditions of cholesterol oxidase treatment were carefully selected to minimize cell permeabilization; an average of 35% of cellular cholesterol was oxidized in six experiments. The specific radioactivity of cholestenone increased rapidly after a 30-min lag in parallel with the increase in specific radioactivity of cholesterol (Fig. 3A and C). The rates of appearance of free [^{3}H]cholesterol in the cell and at the plasma membrane were estimated by determining the maximum specific radioactivities of cholesterol and cholestenone and calculating the rate at which the measured specific radioactivities approached the maximum values over time (Fig. 3B and 3C). The mean half-time for the rate of appearance of free [^{3}H]cholesterol in the cell was 37.0 ± 10.3 min (mean of six experiments, **Table 1**) after the 30-min lag. The mean half-time for the rate of cholesterol transport to the plasma membrane (appearance of [^{3}H]cholestenone) was 41.8 ± 12.2 min

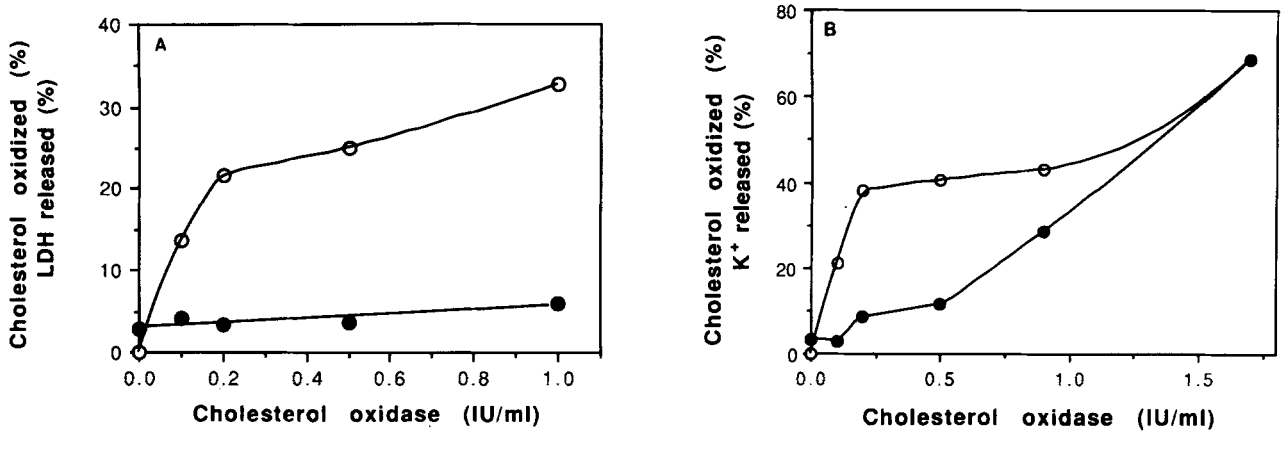
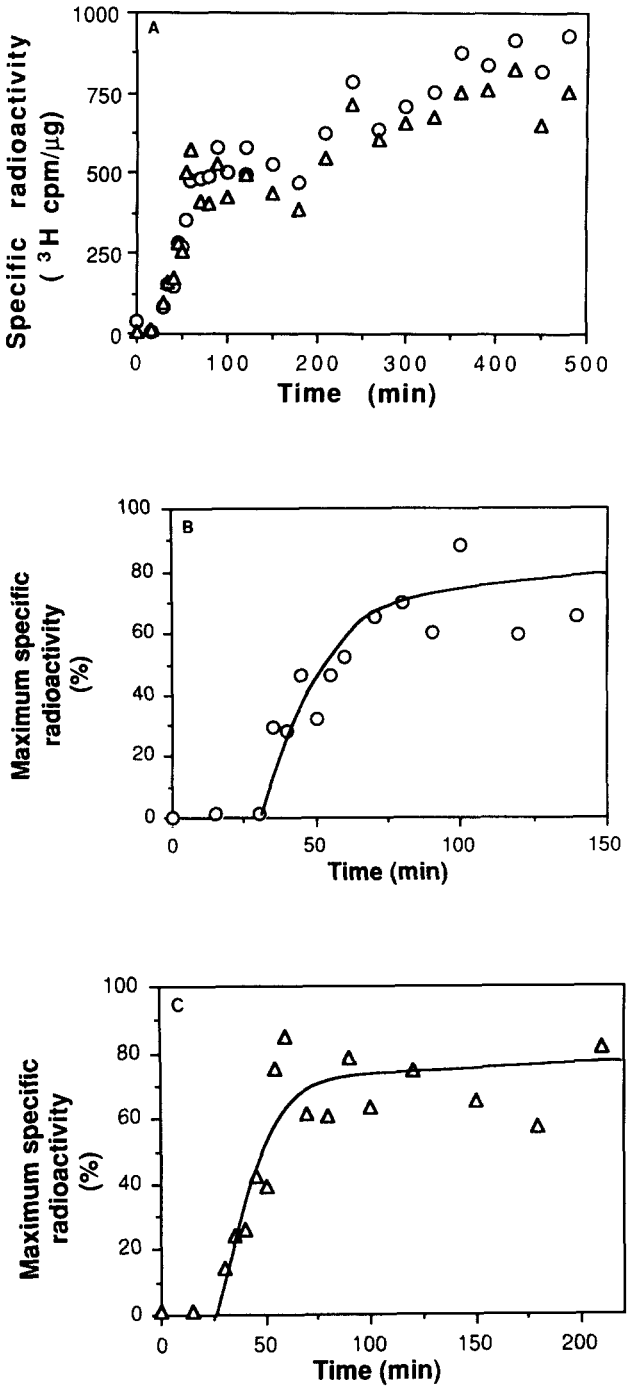


Fig. 2. Cholesterol oxidation and release of LDH activity (A) or intracellular K^+ (B) in adherent cells treated with cholesterol oxidase. Cells on 100-mm dishes were rinsed with PBS and then assay buffer at 25°C. Cholesterol oxidase was added to dishes of cells at the indicated concentrations in assay buffer, and the cells were incubated for 10 min at 25°C. Assay buffer was removed from the cells and LDH activity or K^+ content was determined. Cells were immediately extracted with hexane:isopropanol (3:2), and the lipid content was determined and expressed as described in the legend to Fig. 1. (○) Represents the % cholesterol oxidized, and (●) represents the % of total cellular LDH activity released (A), or the % of total cellular K^+ released (B). Total cellular LDH activity and K^+ were determined as described in Experimental Procedures. The data shown are from one representative experiment out of three (A) and five (B).



(mean of six experiments, Table 1) after the lag. Experiments with an unlabeled LDL chase after the rLDL pulse gave results similar to those from experiments with a lipoprotein-free chase; the transport of radiolabeled cholesterol to the plasma membrane occurred with a half-time of approximately 41 min.

Efflux of [^{3}H]cholesterol from CHO cells treated with [^{3}H]cholesteryl linoleate-labeled rLDL

The rate of [^{3}H]cholesterol efflux from cells incubated with [^{3}H]cholesteryl linoleate-labeled rLDL was determined. The mass of effluxed cholesterol derived from rLDL was calculated from the specific radioactivity of cholesteryl linoleate in rLDL. A 2 h lag was observed before significant [^{3}H]cholesterol appeared in the medium (**Fig. 4**). After the lag, rLDL cholesterol effluxed into the medium at a rate of approximately 0.3 ng rLDL cholesterol/h per μg total cellular free cholesterol (determined in three experiments). By 10 h, approximately 10% of the internalized rLDL cholesterol had effluxed into the incubation medium as free cholesterol when LDL was either present or absent in the chase medium.

Cell permeability measurements

Cells treated briefly with cholesterol oxidase for the rLDL pulse-chase experiments remained intact during enzyme treatment; this was demonstrated by the quantitation of either LDH or K^+ released into the assay buffer (in five out of six experiments). In one experiment, less than 2% of cellular LDH activity was released into the assay buffer from cells treated with cholesterol oxidase above the release of cellular LDH in the no-enzyme controls (data not shown). In four experiments, less than 3% of cellular K^+ was released into the assay buffer by enzyme treatment relative to the release measured in the no-enzyme controls (data not shown).

DISCUSSION

We present the novel observation that LDL cholesterol is very rapidly transported from lysosomes to the plasma membrane of CHO cells. The rate of transport of rLDL

Fig. 3. Release of lipoprotein cholesterol into the cell and incorporation into the plasma membrane. Subconfluent dishes of cells were preincubated under conditions to deplete cellular cholesterol and inhibit endogenous synthesis of cholesterol. Cells were then incubated with [^{3}H]cholesteryl linoleate rLDL at 4°C for 2.5 h and then rinsed with PBS. Chase medium (lipoprotein-deficient medium, four experiments (data shown); medium with 40 μg unlabeled LDL protein/ml, two experiments) was added and cells were incubated at 37°C for the indicated times. Cells were rinsed with PBS and then assay buffer at 25°C. Cholesterol oxidase was added to the cells in assay buffer (0.04–0.2 IU enzyme/ml), and the cells were incubated for 20–30 sec at 25°C. Assay buffer was removed from the cells and released LDH activity or K^+ was determined; less than 2% of cellular LDH activity (one experiment) or less than 3% of cellular K^+ (four experiments) was released into the assay buffer above the same measurements in the no-enzyme controls. Cells were immediately extracted and the lipids were quantitated. (A) Data for release of free cholesterol from rLDL cholesteryl linoleate (○) and transport of cholesterol to the plasma membrane (cholestenone) (△) collected over 8 h. (B) Data from (A) replotted for the release of free cholesterol from rLDL cholesteryl linoleate to show initial rate of cholesterol release as % of maximum specific radioactivity of free cholesterol. (C) Data from (A) replotted for the transport of cholesterol to the plasma membrane to show initial rate of cholesterol transport as % of maximum specific radioactivity of cholestenone. Data shown are from one representative experiment out of six.

TABLE 1. Rates of lysosomal cleavage of rLDL cholesteryl linoleate (cholesterol) and transport of released cholesterol to the plasma membrane (cholestenone)

Experiment	Chase	Cholesterol		Cholestenone	
		$t_{1/2}$	r^2	$t_{1/2}$	r^2
		min		*min*	
1	LDS[a]	27.2	0.95	52.5	0.90
2	LDS	30.6	0.89	38.5	0.98
3	LDS	48.7	0.94	49.1	0.84
4	LDS	32.6	0.83	28.4	0.88
5	LDL[b]	51.4	0.88	55.1	0.90
6	LDL	31.6	0.89	27.1	0.88
Mean		37.0 ± 10.3		41.8 ± 12.2	

[a]Lipoprotein-deficient serum-containing medium.
[b]Low density lipoprotein-containing medium (50 μg/ml LDL protein).

[^{3}H]cholesterol to the plasma membrane was determined using cholesterol oxidase-catalyzed modification of plasma membrane cholesterol under carefully defined conditions. The release of free [^{3}H]cholesterol into cells by lysosomal cleavage of rLDL [^{3}H]cholesteryl linoleate had a half-time of 37 min following a 30-min lag. The appearance of [^{3}H]cholesterol in the plasma membrane had a similar half-time of 42 min following the same lag. The difference between the rate of free cholesterol release from rLDL cholesteryl ester and the rate of appearance of rLDL cholesterol in the plasma membrane was within experimental error of the measurements, suggesting very rapid transport of [^{3}H]cholesterol to the plasma membrane after cleavage of [^{3}H]cholesteryl linoleate. The 30-min lag observed before the appearance of free cholesterol in the cell probably represents the temperature equilibration of cells from 4°C to 37°C and the internalization of rLDL via the LDL cell surface receptor pathway, with subsequent transport of the receptor-ligand complex through the endocytotic pathway and delivery of rLDL to the lysosome, as described previously (reviewed in ref. 35). These observations suggest that internalization and cleavage of cholesteryl esters is the rate-limiting step of LDL cholesterol transport to the plasma membrane.

Cholesterol oxidase has been used in several studies to quantitate plasma membrane cholesterol in various cell lines (29, 36–38). A primary assumption of the use of cholesterol oxidase as a probe for plasma membrane cholesterol has been that the enzyme cannot enter the cell, and therefore acts exclusively on the cell's outer membrane. In all previous studies, glutaraldehyde was used as a fixative prior to treatment of cells with cholesterol oxidase to ensure cell integrity during enzyme treatment (29). We tested the integrity of CHO cells during cholesterol oxidase treatment under the assay conditions of Lange and Ramos (29). While the extent of cellular cholesterol oxidation in our experiments was similar to published results, we also found extensive release of intracellular K^+ during the enzyme treatment. Our results suggest that cells may be permeabilized by cholesterol oxidase treatment under the conditions described previously (29). Furthermore, glutaraldehyde fixation may not protect cells from permeabilization by cholesterol oxidase treatment, since the extents of cholesterol oxidation and K^+ release were similar for cells treated with cholesterol oxidase under the same conditions but without prior glutaraldehyde fixation.

Levels of plasma membrane cholesterol determined by cholesterol oxidase susceptibility of cellular cholesterol are controversial in light of estimates obtained by other methods (11, 39). Cholesterol oxidase accesses greater than 80% of cellular cholesterol in human skin fibroblasts (29, 36), CHO cells, rat hepatocytes (29), rat ovarian granulosa cells (37), and J774 macrophages (38). Mea-

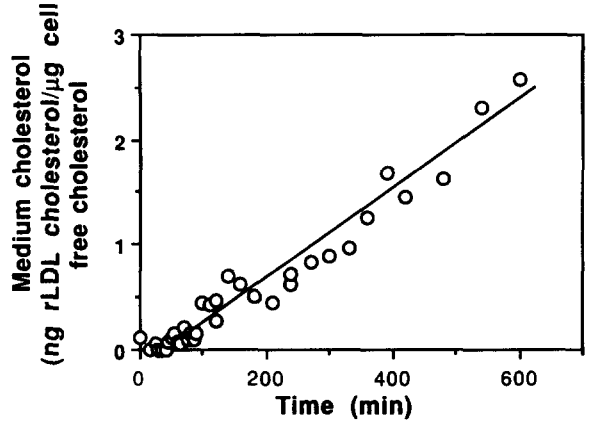


Fig. 4. Efflux of rLDL free cholesterol from cells. Cells were incubated with rLDL as described in the legend to Fig. 3. Chase medium from dishes was removed at the times indicated, and medium cholesterol was extracted and quantitated. The mass of effluxed cholesterol derived from rLDL was calculated from the specific radioactivity of cholesterol in the original rLDL preparation. Data shown are from one representative experiment out of three (one with a lipoprotein-free chase, and two with chase medium containing LDL; data shown).

surements of plasma membrane cholesterol content from subcellular fractionation studies and calculations based on cell surface area have suggested that plasma membrane cholesterol levels in excess of 40% of cellular cholesterol are physically unlikely (11, 39). On the other hand, alternative estimates of plasma membrane surface area and subcellular fractionation studies suggest that 90% of cellular cholesterol is located in the plasma membrane (40). While our studies do not address the question of how much cholesterol is in the plasma membrane, they suggest that susceptibility of cellular cholesterol to cholesterol oxidase may not provide an acccurate estimate of total plasma membrane cholesterol mass. Since extensive oxidation of cellular cholesterol was accompanied by cell permeabilization, as shown by release of cellular K^+, it is possible that cholesterol oxidase has access to intracellular pools of cholesterol as well as plasma membrane cholesterol under these conditions.

Our assay for the appearance of lipoprotein cholesterol at the plasma membrane used cholesterol oxidase under carefully defined conditions. We observed that very little cell permeabilization occurred in cells treated with cholesterol oxidase when less than 40% of cellular cholesterol was converted to cholestenone. This result suggests that at least 40% of cell cholesterol is in the plasma membrane. To avoid potential oxidation of intracellular cholesterol, we chose assay conditions that oxidize less than 40% of cellular cholesterol in our cholesterol transport experiments. We have assumed that the radiolabeled cholesterol tracer has equal access to all plasma membrane cholesterol pools, although we cannot rule out the possibility that oxidation of less than 40% of cellular cholesterol may identify a subdomain of plasma membrane cholesterol that is available to cholesterol oxidase. Under our conditions, negligible release of cellular K^+ and LDH activity into the assay buffer occurred with cholesterol oxidase treatment, indicating that the cells were intact. By determining the increase in specific radioactivity of plasma membrane cholesterol after the incubation of cells with [^{3}H]cholesteryl linoleate-labeled rLDL, we estimated the rate of transport of LDL cholesterol to the plasma membrane by means that do not require the quantitation of total plasma membrane cholesterol levels and are therefore independent of the oxidation of all plasma membrane cholesterol.

Our modified assay provides a significant improvement in the time resolution of sampling possible with cholesterol oxidase-catalyzed modification of plasma membrane cholesterol. By previous methods (29), the processing time of each sample required at least 1.5 h allowing potential diffusion of cholesterol between membranes, hence limiting the accuracy of rate determinations. The processing of each sample in our assay was complete in less than 2 min, thus allowing frequent sampling (see Fig.3A). This improved resolution was essential to the estimation of the rate of lipoprotein cholesterol transport since the transport of free cholesterol released by lysosomal hydrolysis of cholesteryl esters to the plasma membrane occurred within this 2-min time frame.

Our estimates of the rate of lipoprotein cholesterol transport to the plasma membrane are comparable to rates determined in steroidogenic cells by methods similar to the previously published cholesterol oxidase assay protocol (29). Freeman (36) continuously labeled MA-10 Leydig tumor cells with [^{3}H]cholesteryl linoleate rLDL and then measured the transport of [^{3}H]cholesterol to the plasma membrane using a modification of the cholesterol oxidase assay of Lange and Ramos (29). Adherent cells were hypotonically swollen and glutaraldehyde-fixed before extensive cholesterol oxidase treatment, resulting in the oxidation of 66% of cellular free cholesterol. By these methods, the rate of lipoprotein cholesterol transport to the plasma membrane had a half-time of 31.9 min, very similar to our measured rate of appearance of free cholesterol from lysosomal hydrolysis of cholesteryl esters. Since we have shown that cholesterol transport from the lysosome to the plasma membrane is very rapid, the arrival of cholesterol at the plasma membrane would be indistinguishable from the appearance of intracellular free cholesterol if cholesterol oxidase had access to intracellular cholesterol pools. The inclusion of cell integrity measurements in our study allows a distinction between plasma membrane and intracellular cholesterol.

We measured the rate of [^{3}H]cholesterol efflux from cells incubated with [^{3}H]cholesteryl linoleate-labeled rLDL. A 2-h lag was observed before rLDL cholesterol was detected in the medium; the rate of [^{3}H]cholesterol efflux was linear for at least 10 h after the lag. The initial release of [^{3}H]cholesterol into the medium is a measure of the rate of uptake and processing of the rLDL, transport of cholesterol to the plasma membrane, and release of cholesterol from the cell surface. Since we found that the transport of LDL cholesterol to the plasma membrane is rapid, the rate-limiting step of cholesterol efflux is the release from the cell surface into the medium. Several studies examining cholesterol release from fibroblasts support the conclusions that this process is relatively slow (reviewed in ref. 18).

Free cholesterol derived from LDL cholesteryl esters was very rapidly transported to the plasma membrane; the rate of this process was within the time resolution of our method (2 min). This rate of transport suggests that passive diffusion of cholesterol from lysosomes to the plasma membrane is unlikely, since aqueous diffusion of cholesterol between membranes is relatively slow (18). Our study suggests a catalytic mechanism for cholesterol transport between the lysosome and plasma membrane. Studies in Niemann-Pick Type C fibroblasts lend further support to active transport of cholesterol across lysosomal membranes. These fibroblasts take up and hydrolyze

LDL cholesteryl esters normally (41, 42) but released free cholesterol is poorly esterified by ACAT (42, 43) and does not normally down-regulate cholesterol biosynthesis and LDL receptor activity (41, 42), indicating that it is not transported to other intracellular sites. Lipoprotein-derived free cholesterol appears to accumulate in lysosomes (43), and is not further transported to the plasma membrane (43, 44). These observations suggest that Niemann-Pick Type C cells lack the ability to transport cholesterol out of lysosomes, implying that normal cells possess an active transport mechanism. The nature of this mechanism and its general role in intracellular cholesterol transport between other organelles remains to be demonstrated.

We thank Dr. D. L. Ebert and Ms. P. J. Uelmen for editorial assistance. D. L. B. was supported by an American Heart Association predoctoral fellowship. A. D. A. is an Established Investigator of the American Heart Association and was a Shaw Scholar while this work was carried out.

Manuscript received 5 June 1989 and in revised form 18 August 1989.

REFERENCES

1. Carew, T. E., R. C. Pittman, and D. Steinberg. 1982. Tissue sites of degradation of native and reductively methylated [^{14}C]sucrose-labeled low density lipoprotein in rats: contribution of receptor-dependent and receptor-independent pathways. *J. Biol. Chem.* **257:** 8001–8008.
2. Pittman, R. C., T. E. Carew, A. D. Attie, J. L. Witztum, Y. Watanabe, and D. Steinberg. 1982. Receptor-dependent and receptor-independent degradation of low density lipoprotein in normal rabbits and in receptor-deficient mutant rabbits. *J. Biol. Chem.* **257:** 7994–8000.
3. Kesaniemi, Y. A., J. L. Witztum, and U. P. Steinbrecher. 1983. Receptor-mediated catabolism of low density lipoprotein in man: quantitation using glucosylated low density lipoprotein. *J. Clin. Invest.* **71:** 950–959.
4. Spady, D. K., D. W. Billheimer, and J. M. Dietschy. 1983. Rates of receptor-dependent and independent low density lipoprotein uptake in the hamster. *Proc. Natl. Acad. Sci. USA.* **80:** 3499–3503.
5. Colbeau, A., J. Nachbaur, and P. M. Vignais. 1971. Enzymic characterization and lipid composition of rat liver subcellular membranes. *Biochim. Biophys. Acta.* **249:** 462–492.
6. Meldolesi, J., J. D. Jamieson, and G. E. Palade. 1971. Composition of cellular membranes in the pancreas of the guinea pig. II. Lipids. *J. Cell. Biol.* **49:** 130–149.
7. Renkonen, O., C. G. Gahmberg, K. Simons, and L. Kaariainen. 1972. The lipids of the plasma membranes and endoplasmic reticulum from cultured baby hamster kidney cells (BHK21). *Biochim. Biophys. Acta.* **255:** 66–78.
8. Orci, L., R. Montesano, P. Meda, F. Malaisse-Lagae, D. Brown, A. Perrelet, and P. Vassalli. 1981. Heterogeneous distribution of filipin-cholesterol complexes across the cisternae of the Golgi apparatus. *Proc. Natl. Acad. Sci. USA.* **78:** 293–297.
9. Green, C. 1983. The movement of cholesterol within cells. *Biochem. Soc. Trans.* **11:** 637–639.
10. Yeagle, P. L. 1985. Cholesterol and the cell membrane. *Biochim. Biophys. Acta.* **822:** 267–287.
11. Dawidowicz, E. A. 1987. Dynamics of membrane lipid metabolism and turnover. *Annu. Rev. Biochem.* **56:** 43–61.
12. Wattenberg, B. W., and D. F. Silbert. 1983. Sterol partitioning among intracellular membranes: testing a model for cellular sterol distribution. *J. Biol. Chem.* **258:** 2284–2289.
13. Lange, Y., and H. J. G. Matthies. 1984. Transfer of cholesterol from its site of synthesis to the plasma membrane. *J. Biol. Chem.* **259:** 14624–14630.
14. Poznansky, M. J., and S. Czekanski. 1982. Cholesterol movement between human skin fibroblasts and phosphatidylcholine vesicles. *Biochim. Biophys. Acta.* **685:** 182–190.
15. Robertson, D. L., and M. J. Poznansky. 1985. The effect of non-receptor-mediated uptake of cholesterol on intracellular cholesterol metablism in human skin fibroblasts. *Biochem. J.* **232:** 553–557.
16. Sleight, R. G. 1987. Intracellular lipid transport in eukaryotes. *Annu. Rev. Physiol.* **49:** 193–208.
17. Dawidowicz, E. A. 1987. Lipid exchange: transmembrane movement, spontaneous movement, and protein-mediated transfer of lipids and cholesterol. *Curr. Top. Membr. Transp.* **29:** 175–202.
18. Phillips, M. C., W. J. Johnson, and G. H. Rothblat. 1987. Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochim. Biophys. Acta.* **906:** 223–276.
19. Dempsey, M. E., P. S. Hargis, D. M. McGuire, A. McMahon, C. D. Olson, L. M. Salati, S. D. Clarke, and H. C. Towle. 1985. Role of sterol carrier protein in cholesterol metabolism. *Chem. Phys. Lipids.* **38:** 223–237.
20. Vahouny, G. V., R. Chanderbhan, A. Kharroubi, B. J. Noland, A. Pastuszyn, and T. J. Scallen. 1987. Sterol carrier and lipid transfer proteins. *Adv. Lipid Res.* **22:** 83–113.
21. Kaplan, M. R., and R. D. Simoni. 1985. Transport of cholesterol from the endoplasmic reticulum to the plasma membrane. *J. Cell Biol.* **101:** 446–453.
22. Lange, Y., and T. L. Steck. 1985. Cholesterol-rich intracellular membranes: a precursor to the plasma membrane. *J. Biol. Chem.* **260:** 15592–15597.
23. Lange, Y., and M. F. Muraski. 1987. Cholesterol is not synthesized in membranes bearing 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **262:** 4433–4436.
24. Lange, Y., and M. F. Muraski. 1988. Topographic heterogeneity in cholesterol biosynthesis. *J. Biol. Chem.* **263:** 9366–9373.
25. Patel, K. M., L. A. Sklar, H. J. Currie, H. J. Pownall, J. D. Morrisett, and J. T. Sparrow. 1979. Synthesis of saturated, unsaturated, spin-labeled, and fluorescent esters: acylation of cholesterol using fatty acid anhydride and 4-pyrrolidinopyridine. *Lipids.* **14:** 816–818.
26. Lindgren, F. T., L. C. Jensen, and F. T. Hatch. 1979. The isolation and quantitative analysis of serum lipoproteins. *In* Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism. G. J. Nelson, editor. Robert E. Krieger Publishing Co. Huntington, New York. 181–274.
27. Krieger, M. 1986. Reconstitution of the hydrophobic core of low-density lipoprotein. *Methods Enzymol.* **128:** 608–613.
28. 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.
29. Lange, Y., and B. V. Ramos. 1983. Analysis of the distribution of cholesterol in the intact cell. *J. Biol. Chem.* **258:** 15130–15134.
30. Brown, M. S., and J. L. Goldstein. 1980. Multivalent feedback regulation of HMG CoA reductase, a control mech-

anism coordinating isoprenoid synthesis and cell growth. *J. Lipid Res.* **21:** 505–517.
31. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
32. Cleland, W. W. 1979. Statistical analysis of enzyme kinetic data. *Methods Enzymol.* **63:** 103–138.
33. Decker, L. A., editor. 1977. Worthington Enzyme Manual. Worthington Biochemical Corp. Freehold, New Jersey. 19–22.
34. Brasaemle, D. L., A. D. Robertson, and A. D. Attie. 1988. Transbilayer movement of cholesterol in the human erythrocyte membrane. *J. Lipid Res.* **29:** 481–489.
35. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science.* **232:** 34–47.
36. Freeman, D. A. 1987. Cyclic AMP-mediated modification of cholesterol traffic in Leydig tumor cells. *J. Biol. Chem.* **262:** 13061–13068.
37. Lange, Y., V. M. Schmit, and J. R. Schreiber. 1988. Localization and movement of newly synthesized cholesterol in rat ovarian granulosa cells. *Endocrinology.* **123:** 81–86.
38. Tabas, I., W. J. Rosoff, and G. C. Boykow. 1988. Acyl coenzyme A:cholesterol acyl transferase in macrophages utilizes a cellular pool of cholesterol oxidase-accessible cholesterol as substrate. *J. Biol. Chem.* **263:** 1266–1272.
39. Van Meer, G. 1987. Plasma membrane cholesterol pools. *Trends Biochem. Sci.* **12:** 375–376.
40. Lange, Y., M. H. Swaisgood, B. V. Ramos, and T. L. Steck. 1989. Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. *J. Biol. Chem.* **264:** 3786–3793.
41. Pentchev, P. G., M. E. Comly, H. S. Kruth, T. Tokoro, J. Butler, J. Sokol, M. Filling-Katz, J. M. Quirk, D. C. Marshall, S. Patel, M. T. Vanier, and R. O. Brady. 1987. Group C Niemann-Pick disease: faulty regulation of low density lipoprotein uptake and cholesterol storage in cultured fibroblasts. *FASEB J.* **1:** 40–45.
42. Liscum, L., and J. R. Faust. 1987. Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Niemann-Pick Type C fibroblasts. *J. Biol. Chem.* **262:** 17002–17008.
43. Sokol, J., E. J. Blanchette-Mackie, H. S. Kruth, N. K. Dwyer, L. M. Amende, J. D. Butler, E. Robinson, S. Patel, R. O. Brady, M. E. Comly, M. T. Vanier, and P. G. Pentchev. 1988. Type C Niemann-Pick disease: lysosomal accumulation and defective intracellular mobilization of low density lipoprotein cholesterol. *J. Biol. Chem.* **263:** 3411–3417.
44. Liscum, L., R. M. Ruggiero, and J. R. Faust. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick Type C fibroblasts. *J. Cell Biol.* **108:** 1625–1636.