Cell-free transfer of cholesterol from lysosomes to phospholipid vesicles

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Abstract The objective of this work was to develop a cell-free system for studying the transfer of cholesterol from lysosomes to membrane acceptor particles. The methods involved: *1)* loading of CHO cells at 15°C with [^{3}H]cholesteryl oleate-reconstituted LDL, such that it accumulated undegraded in endosomes; *2)* homogenization of cells, followed by preparation of an endosome-lysosome donor fraction; *3)* incubation of the donor fraction at 37°C in a defined cytosol-like medium containing acceptor particles of egg phosphatidylcholine small unilamellar vesicles (PC-SUV); and *4)* measurement of cholesteryl oleate (CO) hydrolysis and transfer of the resulting free cholesterol (FC) to vesicles. During cell-free incubation, LDL-loaded endosomes fused with lysosomes leading to the lysosomal hydrolysis of LDL cholesteryl ester. Maximal hydrolysis of approximately 50% was achieved in 4–8 h. This hydrolysis was inhibited by lysosomotropic agents, proton ionophores, or removal of ATP and GTP from the medium, indicating that it took place in sealed lysosomes. In the absence of PC-SUV, the release of LDL-derived FC from lysosomes was ≤ 10%/8 h. This was increased to a maximum of 25–30%/8 h at 3 mg/ml of PC-SUV. In contrast, the release of undegraded CO was 5–15%/8 h and not stimulated by PC-SUV, suggesting that the transfer of FC to PC-SUV was selective and not due to the uncontrolled release of lysosomal contents. In comparisons between CHO-K1 cells and sterol transport-defective CHO(2-2) cells, lysosomes from the latter cell were 35% less efficient as donors of cholesterol for transfer to egg phosphatidylcholine small unilamellar vesicles, indicating that these methods reproduce an important aspect of sterol trafficking in cells. In addition, this result suggests that the mutation in CHO(2-2) has a direct effect on the lysosomes of these cells.—**Johnson, W. J.** Cell-free transfer of cholesterol from lysosomes to phospholipid vesicles. *J. Lipid Res.* 1996. **37:** 54–66.

Supplementary key words Chinese hamster ovary cell • CHO-K1 • CHO(2-2) • endosome • low density lipoprotein • cholesteryl oleate • free cholesterol • acid cholesteryl ester hydrolase • acid lipase • chloroquine • methylamine • monensin • nigericin • ATP • GTP • calcium ion • N-acetyl-β-glucosaminidase • alkaline phosphodiesterase • cytochrome C oxidase • U18666A • progesterone • imipramine • phosphatidylcholine • small unilamellar vesicles

Cholesterol that is generated in lysosomes from the degradation of LDL is transported to several other cellular locations, including the plasma membrane, rough endoplasmic reticulum (RER), and mitochondria (1, 2). Studies addressing this issue have defined the kinetics of lysosomal sterol transport in cells and have demonstrated its disruption by drugs and genetic mutation. Transport to the RER and mitochondria appears to involve preliminary transit through the plasma membrane (3, 4), suggesting that there are distinct pathways of sterol trafficking in the cell, and that transport from lysosomes to the plasma membrane may be essential for the efficient distribution of exogenous cholesterol throughout the cell. There are some indications that cholesterol transport may be specifically mediated, such as the Niemann-Pick C metabolic disease [which appears to be due to a defect in the release of cholesterol from lysosomes and/or the Golgi apparatus (5)], the isolation of mutant CHO cells that resemble cells from Niemann-Pick C patients (6), and the dependence of lysosomal cholesterol movement to the RER on cytoplasmic vimentin filaments (7). Despite the indications of mediation, the delivery of lysosomal cholesterol to the plasma membrane is not dependent on metabolic energy (8). In addition, the existence of a variety of lysosomal storage diseases caused by the absence of specific acid hydrolases (9) implies that in most cells intermembrane vesicle trafficking from lysosomes to the cell surface is quite limited and probably not extensive enough to account for the known rapid movement of LDL-derived cholesterol to the plasma membrane (10, 11). The latter observations imply that the transport of sterol from lysosomes

Abbreviations: APDase, alkaline phosphodiesterase; BSA, bovine serum albumin; CHO, Chinese hamster ovary (cells); CE, cholesteryl ester; CO, cholesteryl oleate; DLP, delipidized (bovine) serum protein; FBS, fetal bovine serum; FC, free (unesterified) cholesterol; LDL, low density lipoprotein; NABGase, N-acetyl-β-glucosaminidase; PC, egg phosphatidylcholine; PC-SUV, small unilamellar vesicles prepared from egg phosphatidylcholine; r[^{3}H-CO]LDL, low density lipoprotein reconstituted with [^{3}H]cholesteryl oleate; RER, rough endoplasmic reticulum; U18666A, Upjohn compound 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one.

to other organelles may involve some form of intermembrane sterol diffusion, rather than intermembrane vesicle trafficking. The precise mechanism(s) involved in this transport still await clear definition.

Progress toward understanding lysosomal sterol transport would be enhanced by the availability of appropriate cell-free experimental systems. Such systems, in analogy to those already described for studying the cellular transport of proteins and complex lipids (12), would allow direct examination of the transport mechanisms and their possible regulation. The objective of the present studies was to develop a cell-free system for studying the transfer of cholesterol from intact lysosomes to membrane acceptor particles. In the system described here, the sterol donor consisted of lysosomes isolated from CHO cells that had been preloaded with LDL containing [^{3}H]cholesteryl oleate, and the acceptor consisted of egg phosphatidylcholine small unilamellar vesicles (PC-SUV). The methods were such that LDL was degraded during cell-free incubation, rather than during the loading phase when cells were still intact. As described in Results, this approach entailed some heterogeneity in the donor, but had the advantages of providing a direct indication of whether lysosomes were metabolically active and of ensuring that all LDL-derived free cholesterol originated in well-sealed lysosomes. The use of the simple PC-SUV acceptor was expected to confine the transfer mechanism to unmediated diffusion between the donor and acceptor membranes, the simplest of the possible mechanisms operating in cells. This was judged to be a reasonable starting point for the characterization of this system. In addition, it allowed us to address the question of which of the known properties of lysosomal cholesterol trafficking might be attributed to intermembrane sterol diffusion. The results show efficient degradation of LDL within sealed lysosomes during cell-free incubation, transfer of the resulting free cholesterol (FC) to PC-SUV, and a significant reduction in this transfer when the lysosomes were derived from a mutant CHO cell line defective in the transport of lysosomal cholesterol.

MATERIALS AND METHODS

Reagents, biologicals, and cell lines

Unless noted otherwise, chemical and radioisotopic reagents, lipoproteins, delipidized serum protein (DLP), and cell culture supplies were obtained as previously described (11, 13). LDL was reconstituted with a core of [^{3}H]cholesteryl oleate by the potato starch method of Krieger et al. (14), with minor changes as noted previously (11). This product is abbreviated r[^{3}H-CO]LDL. Sucrose and glucose were from Fisher Scientific (Pittsburgh, PA). Potassium salts, EGTA, ATP, GTP, dithiothreitol, imipramine, progesterone, egg phosphatidylcholine, hexokinase, and substrates for spectrophotometric enzyme assays were from Sigma (St. Louis, MO). Creatine phosphate and creatine kinase were from Boehringer Mannheim (Indianapolis, IN). CHO-K1 cells were from the American Type Culture Collection (Rockville, MD). Transport-defective CHO(2-2) cells were provided by Dr. Laura Liscum (Tufts University, Boston, MA).

Preparation of vesicle acceptor particles

Egg phosphatidylcholine small unilamellar vesicles (PC-SUV) were prepared by sonication (15) in 0.15 M KCl. The resulting sonicate was centrifuged at 160,000 *g* for 2 h at 4°C to remove titanium particles and any multilamellar vesicles, and then the supernatant was dialyzed against sucrose-EGTA homogenization buffer (see below), and filter-sterilized before use. The phospholipid content of this preparation was measured colorimetrically by the method of Sokoloff and Rothblat (16).

Preparation of cells and loading with labeled LDL

For routine propagation, monolayer cultures of CHO cells were grown at 37°C in T25 flasks that contained 10 ml of Ham's F12 medium supplemented with 7.5% heat-inactivated fetal bovine serum and 50 μg/ml of gentamicin. For experiments, a confluent monolayer in a T25 flask (approximately 5×10^6 cells) was dispersed with a solution of trypsin and EDTA, diluted into 160 ml of the above growth medium, and then distributed into ten 100-mm diameter culture plates. After 2–3 days growth at 37°C, the medium was removed and 15 ml of fresh medium was applied. Two days before loading with labeled LDL, the growth medium was removed, the cells were rinsed briefly with Ham's F12 medium, and then 12 ml per plate of medium consisting of 5 mg/ml of bovine delipidized serum protein (DLP) dissolved in Ham's F12 was applied. This lipid-poor medium was used to up-regulate cell-surface LDL receptors. In some experiments, this medium was supplemented with tracer [^{14}C]cholesterol (55 Ci/mol), dispersed with ethanol (0.1% final concentration in medium) and egg phosphatidylcholine (5 μg/ml), to label bulk cellular cholesterol. When pre-labeled in this way, cells were exposed to the tracer for approximately 30 h, and then rinsed and incubated overnight in unlabeled DLP medium to allow equilibration of the tracer among cellular pools. After 2 days incubation with DLP, cells were loaded with labeled LDL by first rinsing with Ham's F12 medium and then incubation for 5 h at 15°C in 8 ml per plate of medium consisting of Ham's F12 supplemented with 0.2% bovine serum albumin and 2.5–10 μg protein per

ml of r[^{3}H-CO]LDL (sp act of [^{3}H]CO = 0.1–0.2 mCi/mg). At 15°C, LDL was expected to accumulate in early endosomes and was not subject to degradation (11, 17). After 5 h, the cells were rinsed extensively at 4°C, and then to provide for translocation of LDL into late endosomal vesicles that would be able to fuse efficiently with lysosomes (18), the cells were warmed to 37°C for 5 min in medium containing 0.2% BSA, and then immediately re-rinsed with Ham's F12 medium at 4°C. Preliminary experiments indicated that this brief warm-up resulted in little direct LDL degradation but significantly enhanced the degradation obtained during subsequent cell-free incubation (data not shown).

Homogenization of cells and preparation of endosome-lysosome donor fraction

Homogenization and fractionation of the cells were performed at 0–4°C. For homogenization, cells first were suspended by scraping in 2 ml per plate of divalent cation-free PBS. The cells from 5–10 identically treated plates were pooled and sedimented by centrifugation at 300 *g* for 10 min. The resulting cell pellet was resuspended in 4.5 ml of homogenization buffer (0.25 M sucrose, 2 mM potassium-EGTA, pH 7.0), and then the cells were disrupted by 4–6 passages of the cell suspension through a stainless steel ball bearing homogenizer (19), constructed with a bore of 6.3 mm and using a carbide ball that provided a total clearance of 41 μm. The homogenizer was obtained from Berni-Tech Engineering (Saratoga, CA). Homogenization was judged adequate when microscopic examination indicated that greater than 70% of the cells were disrupted with nuclei still intact. The post-nuclear supernatant was prepared by centrifugation of the homogenate at 1000–1500 *g* for 10 min. The donor fraction was prepared from the post-nuclear supernatant by sedimentation at 10,000 *g* for 60 min. The resulting 10,000 *g* particle fraction was gently resuspended (by repeated passage through the orifice of a Pasteur pipette) in homogenization buffer to a concentration of 2 plate-equivalents per ml (= approximately 3 × 10^7 cell-equivalents per ml) and stored on ice for no longer than 1 h before use in experiments. Analysis of this donor fraction for organellar markers is given in Results.

Cell-free incubation conditions and measurement of cholesteryl ester hydrolysis and cholesterol transfer

Incubations were performed in a total volume of 0.6 ml in screw-cap, round-bottom polypropylene cryopreservation vials. The cell-free incubation medium consisted of 0.125 M sucrose, 19 mM each of the potassium salts of glutamic, aspartic, and gluconic acids, 2 mM potassium EGTA, 2.5 mM $MgCl_2$, 5 mM HEPES buffer, 1 mM each of ATP, GTP, and dithiothreitol, 8 mM creatine phosphate, 50 μg/ml of creatine kinase, and 0.1% (w/v) bovine serum albumin (BSA), with the pH adjusted to 7.0 with KOH or HCl as needed. This medium was adapted from Bennett, Wandinger-Ness, and Simons (20) for examining the translocation of biosynthetic phospholipids to the cell surface in plasma-membrane perforated cells. The concentration of the donor fraction during incubations was approximately 1 × 10^7 cell equivalents per ml. The acceptor consisted of PC-SUV, usually at a concentration of 1 mg/ml. The reason for the inclusion of BSA in the incubation medium was that preliminary experiments had indicated that this addition prevented the nonspecific adherence of the donor fraction to the walls of the incubation vials (data not shown). Incubations were performed in a 37°C water bath with rotary shaking at approximately 150 rpm, usually for a period of 8 h. During incubation at 37°C, endosome-lysosome fusion was expected to occur, leading to lysosomal hydrolysis of LDL [^{3}H]CO and transfer of the resulting [^{3}H]FC to PC-SUV acceptor particles. Incubations were initiated by addition of the donor fraction to the other components of the system in an incubation vial at 4°C, followed immediately by transfer of the capped vials to the 37°C water bath. Incubations were ended by rapid chilling of the incubation vials in an ice-water bath, the removal of two 100-μl aliquots for determination of total isotope recovery and the extent of cholesteryl ester hydrolysis, and centrifugation of the remaining 400 μl of the medium at 13,600 *g* for 30 min at 4°C followed by removal of two 100-μl aliquots of the supernatant for determination of the total release of isotope from lysosomes and the distribution of the released tracer between free and esterified forms. In a typical experiment, greater than 90% of undegraded [^{3}H]CO remained sedimentable and the recovery of acceptor PC-SUV in the nonsedimentable portion of the system was 86% or greater. Thus, the separation of donor and acceptor by centrifugation was effective, and there was little apparent aggregation of donor and acceptor particles.

Radioactivity was quantified by liquid scintillation counting. The counting efficiencies for ^{14}C and ^{3}H were approximately 80% and 45%, respectively. To determine the distribution of radioactivity between free and esterified cholesterol, lipids were extracted by the procedure of Bligh and Dyer (21), and then free and esterified sterols were separated on silica-impregnated fiberglass ITLC-SA sheets (Gelman, Ann Arbor, MI) using a solvent of petroleum ether and toluene (65:35, v/v) (13). Plate sections corresponding to free and esterified cholesterol were combined directly with liquid scintillant for scintillation counting. Results were calculated in cpm units, and then in most cases converted to units of fractional hydrolysis of cholesteryl ester or fractional

TABLE 1. Recovery of biochemical and organellar markers in the endosome-lysosome donor fraction

A:

r[^{3}H-CO]LDL	[^{14}C]Cholesterol	NABGase	APDase
% recovery in 10,000 g donor fraction vs. PNS			
81 ± 6 (n = 10)	72 ± 7 (n = 9)	76 ± 13 (n = 3)	76 ± 9 (n = 2)

B:

NABGase	APDase	Cytochrome c Oxidase	Protein	Phospholipid	Cholesterol
% recovery in 10,000 g donor fraction vs. PNS					
82 ± 2	66 ± 2	107 ± 3	25 ± 1	37 ± 2	69 ± 3

CHO-K1 cells were incubated 1.5 days with DLP + [^{14}C]cholesterol, equilibrated overnight in DLP, and then incubated 5 h at 15°C with r[^{3}H-CO]LDL. The postnuclear supernatant (PNS) and 10,000 g donor fraction were prepared and analyzed for the indicated markers. Data are expressed as percentage recovery of each marker in the 10,000 *g* donor fraction relative to its recovery in the PNS. The markers correspond to organelles as follows: LDL, endosomes; cholesterol mass and [^{14}C]cholesterol, plasma membrane; NABGase, lysosomes; APDase, plasma membrane; cytochrome c oxidase, mitochondria; phospholipid, total membrane. A: provides data from multiple independent fractionations. B: provides data from a single fractionation that did not involve any radioactive tracers, and in which native human LDL was substituted for r[^{3}H-CO]LDL. For this fractionation, all assays were performed in duplicate or triplicate and the uncertainties indicate the range or standard deviation, respectively.

release of cholesterol from the donor. Results are described in terms of the overall release of cholesterol from the donor fraction, or in some cases in terms of transfer to PC-SUV, which is defined as the portion of release due to the addition of PC-SUV to the incubation system.

Other analytical methods

Cholesterol and phospholipid mass analyses were as described previously (11, 16). Protein was determined by a modification of the Lowry procedure (22), using BSA as the standard. Activities of N-acetyl-β-glucosaminidase (NABGase) and alkaline phosphodiesterase (APDase) were measured in the presence of 0.1% Triton X-100, as described by Harrison and Bowers (23) and Beaufay et al. (24), respectively. Cytochrome c oxidase was assayed as described by Cooperstein and Lazarow (25).

Statistical analysis

All incubations were performed at least in triplicate. Data are expressed as means (± 1 SD) of the replicate determinations. Statistical significance was assessed by Student's *t*-test, with $P < 0.05$ as the criterion of significance.

RESULTS

Composition of donor fraction

In the course of several experiments, the 10,000 *g* donor fraction from CHO-K1 cells was analyzed for the recoveries of several enzymatic and biochemical markers: N-acetyl-β-glucosaminidase (NABGase, a lysosomal enzyme marker), alkaline phosphodiesterase (APDase, an enzyme of the plasma membrane), cytochrome c oxidase (a mitochondrial marker), cholesterol mass and [^{14}C]cholesterol (present largely in the plasma membrane), ^{3}H-labeled LDL (present in endosomes with the incubation methods used in these studies), and phospholipid mass (a general membrane marker). These data are summarized in **Table 1.** The recoveries of the three enzyme markers, cholesterol mass, [^{14}C]cholesterol, and ^{3}H-labeled LDL in the donor fraction were all greater than 66%, indicating that most of the lysosomes and endosomes were recovered in this fraction, but that it also contained substantial amounts of plasma membrane and mitochondria. The recoveries of protein and phospholipid were 25% and 37%, respectively, indicating that despite the heterogenity of the donor fraction, most of the cellular protein and most of the nonlysosomal and nonendosomal membranous organelles were eliminated. In preliminary attempts to prepare a more nearly pure endosome-lysosome donor fraction by sucrose-density gradient centrifugation, we succeeded in eliminating most of the plasma-membrane contamination. However, the resulting preparation was unable to hydrolyze LDL cholesteryl ester (data not shown). Because this suggested significant loss of lysosomal function, we chose to proceed with experiments using the 10,000 *g* membrane fraction as the donor, and relying on the addition of a large excess of PC-SUV acceptor to ensure that the cholesterol released from lysosomes was transferred to the acceptor rather than among cell-derived membranes in the donor fraction. In most experiments the PC-SUV acceptor was used at a concentration of 1 mg/ml. This resulted in a 4- to 5-fold excess of

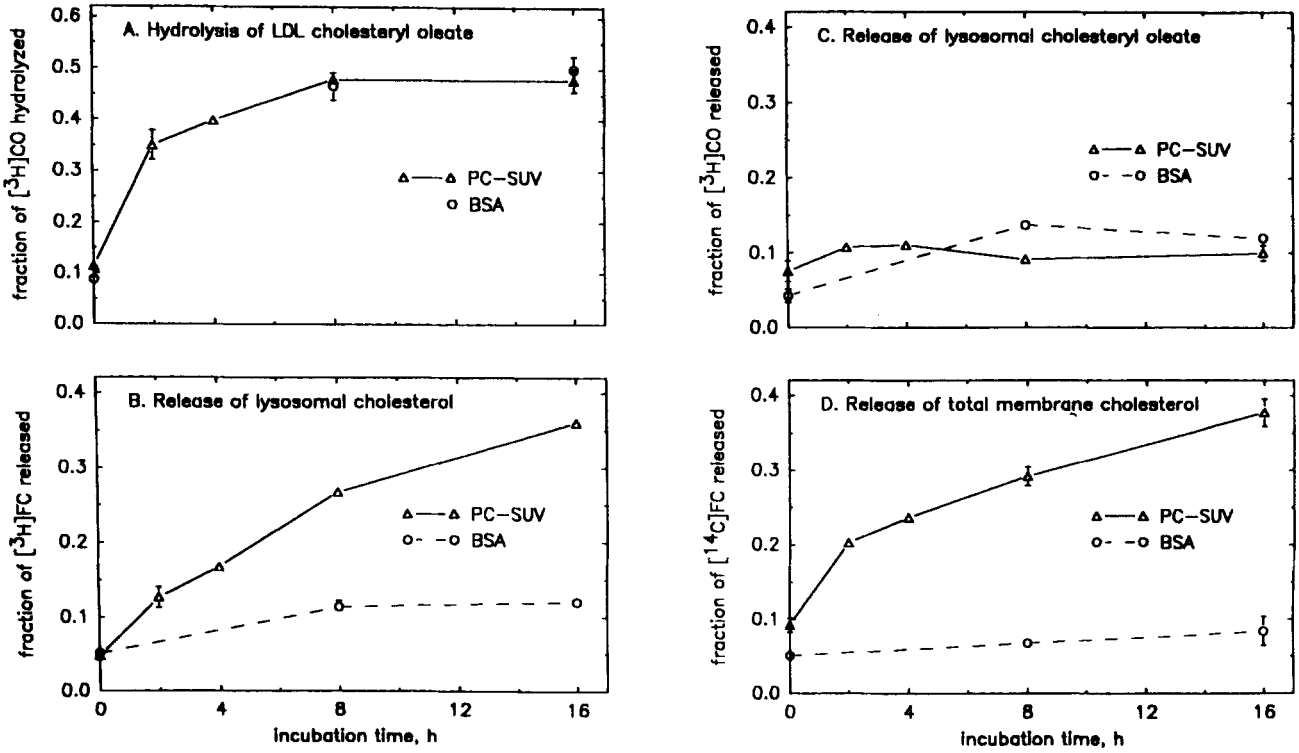

Fig. 1. Time courses of LDL cholesteryl ester hydrolysis and cholesterol release from lysosomes during cell-free incubation of 10,000 *g* donor fraction with egg PC vesicle (PC-SUV) acceptor particles. CHO-K1 cells were labeled with unesterified [^{14}C]cholesterol at 37°C and with r[^{3}H-CO]LDL at 15°C, as described in Materials and Methods. Working at 0–4°C, a cell homogenate was prepared, from which the 10,000 *g* donor fraction was isolated. This was resuspended in cell-free incubation medium containing ATP (1 mM), GTP (1 mM), an ATP-regenerating system, and BSA (0.1%), with or without PC-SUV (1 mg/ml), and then incubated at 37°C for periods of 2–16 h with rotary shaking at 150 rpm. Incubations were ended by cooling the incubation vials in an ice-water bath, taking samples for determination of cholesteryl ester hydrolysis, and then centrifuging the medium at 13,600 *g* for 30 min at 4°C, after which the supernatant was sampled for determination of cholesterol release from the donor. Initially, each incubation contained 7212 ± 128 cpm of [^{3}H]cholesterol (free/total = 0.10 ± 0.02) and 1333 ± 43 cpm of [^{14}C]cholesterol (free/total = 0.96 ± 0.01) Panel A: Time course of LDL cholesteryl ester hydrolysis with PC-SUV acceptor (▲) or without the vesicle acceptor (○). Panel B: Release of LDL-derived [^{3}H]FC from the donor. Fractional release was calculated by normalizing the [^{3}H]FC cpm released at a given time to the total production of [^{3}H]FC at 8 h of incubation. In this way, the fractional release values parallel the cpm release of [^{3}H]FC. Panel C: Release of unhydrolyzed [^{3}H]cholesteryl oleate from the donor. Calculation of fractional release was as described for panel B. Panel D: Release of total membrane [^{14}C]FC from the donor. Fractional release was calculated by normalization to the [^{14}C]FC available at t = 0.

acceptor (phospholipid) in comparison to the donor. As shown in the data below, this concentration of acceptor produced a near maximal rate of lysosomal cholesterol release.

The assertion that LDL was located in endosomes in the starting donor fraction was supported by results of the sucrose density-gradient fractionation mentioned above. These results showed that the LDL label distributed in a broad peak that was intermediate in density between the plasma membrane and the lysosomal enzyme markers (data not shown), as has been observed previously for material within endosomes (26).

Time-courses of lysosomal cholesteryl ester hydrolysis and free cholesterol release

The time courses of LDL cholesteryl ester hydrolysis and free cholesterol release to medium with or without PC-SUV (1 mg/ml) are shown in **Fig. 1.** Important points illustrated by these data are as follows. *1)* Cholesteryl ester hydrolysis achieved a maximal value of about 50% after 8 h of incubation (Fig. 1A). *2)* The release of lysosomal ^{3}H and total membrane [^{14}C]free cholesterol to medium lacking PC-SUV was minimal and not steeply time-dependent (Figs. 1B and D, circles). *3)* The addition of PC-SUV to the medium produced a 2- to 3 fold stimulation of release of lysosomal free cholesterol, and this release was essentially linear with time for at least 8 h (Fig. 1B, triangles). Similar results were obtained with total membrane cholesterol, although the rate of release in the initial 2 h was greater than in subsequent intervals (Fig. 1D). *4)* The release of unhydrolyzed cholesteryl oleate was relatively small, not steeply time-dependent, and not stimulated by the addition of PC-SUV (Fig. 1C), indicating that the release of lysosomal free cholesterol did not represent wholesale release of lysosomal contents. Based on these results,

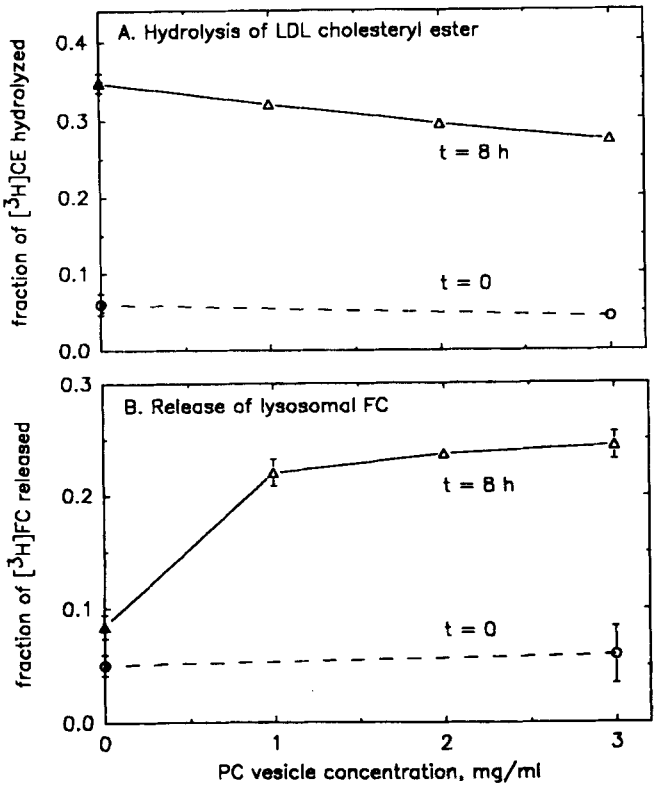

Fig. 2. Dependence of lysosomal cholesterol release on vesicle acceptor concentration. Conditions were as described in Fig. 1, except that the PC-SUV concentration was varied from 0–3 mg/ml and the only non-zero time point was 8 h. Panel A: Hydrolysis of LDL cholesteryl ester as a function of acceptor concentration. Circles, t = 0. Triangles, t = 8 h. Panel B: Release of [^{3}H]FC from lysosomes.

the standard incubation time chosen for most experiments was 8 h. This provided for maximal LDL cholesteryl ester hydrolysis, values of lysosomal free cholesterol release that reflected the initial rate of this process, and minimal release of lysosomal cholesteryl ester.

Dependence of cholesterol release on vesicle concentration, and effect of supplementation with unlabeled donor fraction

To determine the maximal rate of transfer of lysosomal cholesterol to PC-SUV, the acceptor concentration was varied in the range 0–3 mg/ml for a single 8-h time point (**Fig. 2**). The results showed that hydrolysis of LDL cholesteryl ester was inhibited slightly by increasing vesicle concentration (Fig. 2A, triangles). The fractional release of lysosomal free cholesterol rose steeply in the range 0–1 mg/ml of acceptor, and then increased only slightly in the range 1–3 mg/ml (Fig. 2B, triangles). Thus, a near-maximal rate of transfer of lysosomal cholesterol was achieved with an acceptor concentration of 1 mg/ml. For the examination of other variables in subsequent experiments, a PC-SUV concentration of 1 mg/ml was used.

To determine whether nonlysosomal cell-derived membranes in the donor fraction were interfering with cholesterol transfer to PC-SUV and thereby limiting the rate of this transfer, we tested whether the addition of unlabeled 10,000 *g* donor fraction (in an amount equal to the labeled donor) reduced the release of LDL-derived FC to PC-SUV medium. The only effect of this addition was a 4–10% stimulation of the hydrolysis of LDL cholesteryl oleate; there was no effect on the fractional release of cholesterol with either 1 or 3 mg/ml of PC-SUV acceptor (data not shown), suggesting that nonlysosomal membranes in the donor did not interfere significantly with FC transfer to PC-SUV.

Stability of lysosomes in the donor fraction

To examine the stability of lysosomes in the donor fraction, the effects of the incubation conditions on the latency and sedimentability of NABGase were examined. Latency was defined as the percent of total enzyme activity not detectable in the absence of 0.1% Triton X-100. Sedimentability was the percent of activity pelleted by centrifugation at 13,600 *g* for 30 min. The results (**Table 2**), showed that after 8 h incubation with 0 or 1 mg/ml of PC-SUV, NABGase latency declined by about one-third, from an initial value of 67% to final values of 53% and 42%, respectively. Sedimentability showed less change, with initial values of 90–94% and final values of 86–95%. The latency results suggest the development of some leakiness of the lysosomal membrane to low molecular weight solutes. The sedimentability results, however, indicate that NABGase, which is a soluble enzyme of the lysosol (27), was effectively contained, and thus that the lysosomal membrane remained intact throughout the incubations.

TABLE 2. NABGase latency and sedimentability before and after cell-free incubation

Incubation Time and Conditions	% Latency of NABGase[a]	% Sedimentability of NABGase[b]
Time, 0		
BSA	68 ± 2	94 ± 1
PC-SUV	67 ± 3	90 ± 1
Time, 8 h		
BSA	53 ± 1	95 ± 0.1
PC-SUV	42 ± 2	86 ± 1

Incubation conditions were as described in Fig. 1, except no radioactive tracers were used and native LDL was substituted for r[^{3}H-CO]LDL.

[a]Percentage of activity not detected in the absence of 0.1% Triton X-100 in the NABGase assay cocktail.

[b]Percentage of activity sedimented by centrifugation at 13,600 *g* for 30 min.

TABLE 3. Effects of inhibitors of lysosomal acidification on LDL cholesteryl ester hydrolysis during cell-free incubation

Time and Treatment	% [^{3}H]CO Hydrolyzed	% Inhibition of Hydrolysis
T, 0	9 ± 1	—
T, 8 h		
Control (no EtOH)	33 ± 2	—
Chloroquine, 100 μM	12 ± 2	87
Methylamine, 10 mM	16 ± 2	69
Control (0.1% EtOH)	34 ± 1	—
Monensin, 20 μM	13 ± 1	83
Nigericin, 20 μM	11 ± 2	90

CHO-K1 cells were labeled with r[^{3}H-CO]LDL and then used to prepare the 10,000 *g* donor fraction as described in Materials and Methods. The donor fraction was incubated for 8 h with PC-SUV (1 mg/ml) in the usual cell-free medium with the indicated supplement(s). Samples from the beginning and end of the 8-h period were extracted and analyzed by ITLC to determine the extent of hydrolysis of LDL cholesteryl oleate. Monensin and nigericin were added to the incubations from 1000 × stocks in ethanol (EtOH). Chloroquine and methylamine hydrochloride were added from dry stocks.

Effect of inhibition of lysosome acidification

If the hydrolysis of LDL cholesteryl ester was taking place in intact lysosomes, then it should be sensitive to the inhibition of lysosomal acidification. To test this prediction, the effects of adding different proton ionophores and lysosomotropic agents to the cell-free system were examined (**Table 3**). The ionophores, monensin and nigericin and the lysosomotropic agents chloroquine and methylamine all produced dramatic inhibition of hydrolysis in the range of 69–90%. Under all conditions, the undegraded [^{3}H]CO remained > 90% sedimentable when incubations were ended. Thus, the inhibition of hydrolysis could not be attributed to extensive disruption of the lysosomal membrane. In other experiments, it was found that when r[^{3}H-CO]LDL was added directly to an unlabeled cell homogenate and incubated under the conditions of these experiments, there was no significant hydrolysis of the labeled cholesteryl ester (data not shown). Thus, preloading of the LDL into the endosome-lysosome system was necessary in order to obtain efficient LDL degradation during subsequent cell-free incubation. These observations argue strongly that the degradation of LDL obtained under the conditions of these studies occurred within intact lysosomes.

Effects of removing ATP and GTP from the medium

Fusion of endosomes and lysosomes requires ATP and possibly GTP (18), and the acidification of the lysosomal lumen depends on the ATP-dependent proton pump in the lysosomal membrane (28). In addition, the release of cholesterol from lysosomes might in some way depend on one or both of these nucleotides. For these reasons, it was important to examine the effects of removing ATP and GTP from the cell-free incubation system. The removal of both nucleotides was accomplished by the omission of ATP, GTP, and the ATP-regenerating system from the medium in combination with the addition of an ATP scavenging system (glucose plus hexokinase) to eliminate any residual cell-derived ATP (29). As shown by the results in **Table 4,** these modifications caused a dramatic inhibition of LDL cholesteryl ester hydrolysis (72–75%), and a moderate inhibition of the fractional release of free cholesterol (26% reduction in release to PC-SUV medium). The pattern of effects on FC release was interesting in that for both 0 and 1 mg/ml of PC-SUV acceptor, there were similar absolute reductions in FC release (i.e., the fractional release was reduced 0.05–0.06 in both cases). This result may indicate that the small release of FC normally observed in the absence of PC-SUV resulted from nucleotide-dependent budding of small vesicles (not sedimentable at 13,600 *g*) from the lysosomes. This process was not explored further in these studies but deserves more careful examination.

In a separate experiment, GTP alone was omitted from the incubation medium. In this case, there was a moderate inhibition of hydrolysis (24%), but no effect on the fractional release of FC to PC-SUV medium (data not shown). Thus, the effects described in Table 4 were due mostly to the removal of ATP.

Effects of Ca^{2+} ion

Ca^{2+} ion has been reported to stimulate the fusion of endosomal vesicles (30). In addition, elevated cytosolic Ca^{2+} in cells of the arterial wall has been implicated in the progression of atherosclerosis (31). Therefore, it was of interest to determine the effects of Ca^{2+} ion on the parameters of LDL metabolism being examined in these studies. Previous experiments had involved no deliberate addition of Ca^{2+} salts and the medium contained 2 mM of EGTA, an effective Ca^{2+} chelator. Thus, the free [Ca^{2+}]

TABLE 4. Effect of removing ATP and GTP on cholesteryl ester hydrolysis and cholesterol release from lysosomes

Incubation Time and Conditions	% [^{3}H]CO Hydrolyzed	% [^{3}H]FC Released
Time, 0	8 ± 2	2 ± 2
Time, 8 h		
BSA, control	52 ± 2	6 ± 2
BSA, no ATP and GTP	19 ± 2	1 ± 0.4
PC-SUV, control	40 ± 1	23 ± 2
PC-SUV, no ATP and GTP	17 ± 3	17 ± 3

CHO-K1 cells were prelabeled with r[^{3}H-CO]LDL and used to prepare the 10,000 *g* donor fraction. Control incubation conditions were as described in Fig. 1. To determine the effects of nucleoside triphosphate removal, ATP, GTP, and the components of the ATP-regenerating system were removed and the medium was supplemented with 10 mM glucose and 10 units/ml of hexokinase to scavenge any residual ATP (29). The incubation time was 8 h. Hydrolysis results are expressed as percentage of [^{3}H]cholesterol in the unesterified form. Cholesterol release is expressed as the percentage of [^{3}H]FC that was nonsedimentable when incubations were ended.

would have been essentially zero. For the addition of free Ca^{2+} in a well-controlled way, we used a software package that calculated the total addition of $CaCl_2$ needed to obtain a given level of free Ca^{2+}, taking into account the chelating properties of other medium components. Free Ca^{2+} levels of up to 10^{-5} M were tested (**Fig. 3**). The results indicated a modest stimulation of cholesteryl ester hydrolysis, beginning at Ca^{2+} levels of 10^{-7} M (Fig. 3A), a somewhat more substantial inhibition of free cholesterol release to PC-SUV medium beginning at 10^{-7} to 10^{-6} M (Fig. 3B), and no significant effect on the release of undegraded cholesteryl oleate at any of the tested Ca^{2+} concentrations (Fig. 3C). The addition of Ca^{2+} caused no reduction in the recovery of PC-SUV acceptor in the nonsedimentable portion of the incubation system (data not shown). Thus, the inhibition of cholesterol release with increasing Ca^{2+} could not be attributed to aggregation and sedimentation of PC-SUV with the lysosome donor.

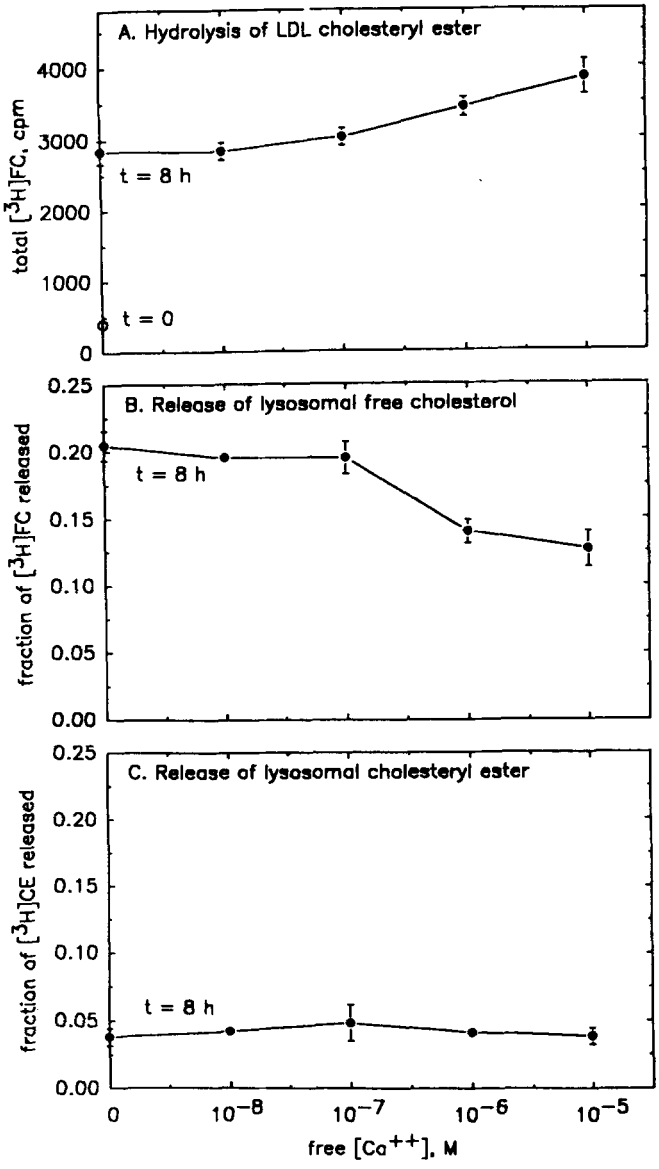

Fig. 3. Effect of Ca^{2+} on cholesteryl ester hydrolysis and cholesterol release from lysosomes. Conditions were as in Fig. 1, except that the only non-zero time point was 8 h, KCl was substituted for the organic potassium salts in the usual incubation media, and media were supplemented with $CaCl_2$ in amounts calculated to yield the indicated free Ca^{2+} concentrations after accounting for chelating properties of other medium components. The substitution of KCl in this experiment was necessary since the program used to calculate free [Ca^{2+}] did not take into account the chelating properties of the organic anions (glutamate, gluconate, and aspartate) in the usual potassium salts mixture. Panel A: Hydrolysis of LDL cholesteryl oleate as a function of free Ca^{2+}. Open circle, t = 0. Closed circles, t = 8 h. Panel B: Release of lysosomal [^{3}H]FC to media containing PC-SUV (1 mg/ml) after 8 h. Panel C: Release of unhydrolyzed lysosomal [^{3}H]cholesteryl oleate.

TABLE 5. Effects of compound U18666A, progesterone, and imipramine on cholesteryl ester hydrolysis and cholesterol release

Treatment	% [^{3}H]CO Hydrolyzed	% [^{3}H]FC Released
Control	34 ± 1	19 ± 3
U18666A	29 ± 1	18 ± 3
Progesterone	32 ± 3	17 ± 6
Imipramine	24 ± 1	18 ± 1

Conditions were as described in Fig. 1, with a single 8-h time point. The PC-SUV concentration was 1 mg/ml. The concentrations of U18666A, progesterone, and imipramine were 1 μg/ml, 10 μg/ml, and 20 μM, respectively; all were added to media from 1000× ethanolic stock solutions. Control medium contained 0.1% ethanol.

Effects of sterol transport inhibitors

Several pharmacological agents have been reported to interfere with the trafficking of lysosomal cholesterol when tested with intact cells. Three of these agents are progesterone (32), the Upjohn experimental androstene derivative U18666A (33), and imipramine (34). It was of interest to determine whether any of these compounds reduced the release of lysosomal cholesterol to PC-SUV medium during cell-free incubation. When tested at concentrations reported to inhibit cholesterol transport in intact-cell experiments, there were some modest effects on the hydrolysis of LDL cholesteryl ester but no significant inhibition of the fractional release of lysosomal cholesterol to PC-SUV medium (**Table 5**). Higher concentrations of U18666A were also tested, but these were found to produce dramatic inhibition of LDL cholesteryl ester hydrolysis (data not shown). Thus, by the direct addition of these three agents to the cell-free system, we could not demonstrate an inhibition of cholesterol release to medium containing PC-SUV.

Comparisons between CHO-K1 and CHO(2-2) lysosomes

CHO-cell variants defective in the transport of lysosomal cholesterol, and thus resembling cells from type C Niemann-Pick patients, have been isolated

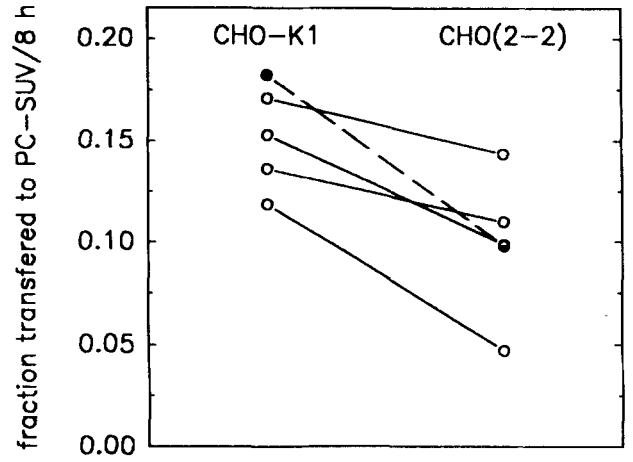

Fig. 4. Cholesterol-transfer comparisons between CHO-K1 and CHO(2-2) lysosomes. This figure summarizes five independent comparisons between CHO-K1 and CHO(2-2) lysosomes using the methods described in the previous figures and tables. Each line indicates the pairing of values in a given experiment. In all cases the incubation time was 8 h. The values are for vesicle-dependent transfer of lysosomal cholesterol in fractional units (i.e., fractional release with 1 mg/ml of PC-SUV in the medium minus release without vesicles). In one experiment (dashed line), the incubations were ended by filtration removal of the lysosomal donor (using 0.1 μm pore-size filters) rather than by centrifugation. Regardless of the inclusion of this experiment in the data analysis, cholesterol transfer from CHO(2-2) lysosomes was significantly less ($P < 0.05$) than from CHO-K1 lysosomes, as assessed by the paired *t*-test.

by Dahl et al. (6) and Cadigan, Spillane, and Chang (35). In the present studies, several cell-free incubation comparisons were made between one of these variants, CHO(2-2) (6), and the fully functional CHO-K1 cells. The results (**Fig. 4**) are expressed in terms of cholesterol transfer to PC-SUV (i.e., the vesicle-dependent component of cholesterol release). Each line in Fig. 4 indicates the pairing of values in a given experiment. Overall, these results suggested a 35% deficit in the ability of the CHO(2-2) lysosomes to transfer cholesterol to PC-SUV. This difference was significant at $P < 0.03$. In some of these experiments, the total membrane cholesterol fraction was labeled with [^{14}C]cholesterol. Transfer of this tracer from CHO(2-2) lysosomes was also reduced, on average by 33% (data not shown). In a time-course study with the CHO(2-2) donor, the release of LDL-derived lysosomal cholesterol to PC-SUV medium was sustained for at least 16 h, similar to the time course for CHO-K1 donor shown in Fig. 1. At 8 and 16 h of incubation, the vesicle-dependent release from the CHO(2-2) lysosomes was 64% and 90%, respectively, of that from the CHO-K1 lysosomes. These results indicate that the reduced transfer of cholesterol from the CHO(2-2) donor is based on a kinetic difference, rather than greater equilibrium retention of cholesterol in the CHO(2-2) lysosomes.

DISCUSSION

Integrity of lysosomes

Several results indicated that the lysosomes remained functional and intact during cell-free incubation. First, the ability to degrade LDL was retained. This indicated that lysosomes were able to fuse with LDL-loaded endosomes, and that the acid lipase in the resulting secondary lysosomes was active. Second, NABGase latency and sedimentability remained high during the incubations. Third, the hydrolysis of LDL cholesteryl ester was sensitive to proton ionophores and lysosomotropic agents that prevent lysosomal acidification. If the limiting membranes of lysosomes had not been intact, there would have been no possibility for the generation of an acidic microenvironment, and the addition of these agents would have been inconsequential to hydrolysis. Thus, the inhibition of hydrolysis by these agents is strong evidence that the lysosomal membranes were intact. Fourth, the removal of ATP and GTP from the medium prevented hydrolysis. This effect probably was due to a combination of preventing lysosome-endosome fusion (which may require both ATP and GTP) (18) and of stopping the function of the ATP-dependent proton pump (28). This result also argues strongly for the integrity of the lysosomes. Finally, the release of undegraded cholesteryl ester from the donor fraction was relatively small and not stimulated by PC-SUV. Thus, the vesicle-stimulated release of free cholesterol could not be attributed to disintegration of the lysosomal membrane leading to general release of lysosomal contents.

Relationship to previous studies on lysosomal LDL metabolism and cell-free reconstitution of lysosome function and lipid transport

Previous studies with intact cells and organs show that the itinerary of LDL degradation involves binding of LDL to its specific cell surface receptor in clathrin-coated pits, followed by endocytosis and sequential association of the lipoprotein with coated vesicles, uncoated vesicles often with tubular extensions (compartment for uncoupling of receptors and ligands, CURL), large multivesicular bodies (late endosomes), and finally perinuclear lysosomes, thought to be formed by the fusion of late endosomes with primary lysosomes (36–39). Previous cell-free studies of endosome-lysosome interaction suggest that the association of late endosomes with lysosomes requires ATP and is stimulated by GTP, and that the mixing of lysosomal and endosomal contents may be dependent on cytosolic components (18, 26). Other studies on endosome-endosome interaction have demonstrated that fusion can occur independently of cytosol and ATP in the presence of micromolar concentrations of free Ca^{2+} (30). Previous

cell-free studies on the hydrolysis of substrates preloaded into rat liver lysosomes or endosomes have demonstrated ATP-dependent degradation of ^{131}I-labeled albumin (40), chloroquine-sensitive hydrolysis of methyl-leucine (41), and lipolysis of VLDL triacylglycerol (42). Studies with isolated fibroblast lysosomes have demonstrated membrane transporters for a variety of water-soluble nutrients, including sugars, amino acids, and nucleosides (43). The mechanisms of sterol and fatty acid release from lysosomes have not been addressed previously. In cell-free studies of biosynthetic lipid transport from liver Golgi apparatus (in suspension) to plasma membrane (immobilized on nitrocellulose strips), it has been reported that transfer was stimulated by NADH, but not by ATP (44).

There is good correspondence between the present results and those obtained in previous studies of lysosome function under cell-free conditions. For instance, in the present studies, LDL degradation showed a strong dependence on nucleoside triphosphates, consistent with requirements for endosome-lysosome fusion and lysosome acidification. Similar to the results of Mego (40), the maximal degradation of entrapped ligand was approximately 50% and achieved within a few hours of incubation. In addition, the degradation was sensitive to lysosomotropic agents, as described by Reeves (41) for the hydrolysis of methyl-leucine. One possible discrepancy between our results and previous data is that we obtained efficient LDL degradation in the absence of cytosol, which may be important for efficient mixing of endosome-lysosome contents using liver-derived organelles (26). One possible explanation for this result is that the degradation of LDL observed in the present studies occurred in endosomes, rather than in secondary lysosomes formed by endosome-lysosome fusion. This seems unlikely based on the data of Runquist and Havel (38) showing that acid cholesteryl ester hydrolase is present in endosomes but exhibits little activity against endocytosed LDL, due to the inability of endosomes to reach a sufficiently low pH. Alternatively, the lack of requirement for cytosol in the present studies may indicate that under some conditions of cellular fractionation the cytosolic proteins needed for endosome-lysosome fusion remain associated with these organelles. In addition, the retention of such factors may depend on the species or tissue source of organelles.

Factors limiting cholesterol transfer to PC-SUV

The maximal rate of release of lysosomal cholesterol to PC-SUV medium was approximately 25% in 8 h, or 3% per h, indicating a half-time for transfer to PC-SUV of at least 23 h (Fig. 2). In contrast, with intact cells the rate of cholesterol transport to the plasma membrane has a half-time of 50 min or less (10, 11), suggesting a rate constant for transport ≥ 80% per h. Thus, the cell-free transfer of cholesterol from lysosomes to PC-SUV was substantially slower than sterol transport in intact cells. The slow transfer under cell-free conditions probably was not due to lack of sufficient acceptor to solubilize cholesterol. This explanation is unlikely based on calculations showing that the hydrolysis of LDL cholesteryl ester in a typical experiment was about 60 pmol per incubation, which was far less than the 775 nmol of PC available in the vesicle acceptor when its concentration was 1 mg/ml. Likewise, the slow transfer of lysosomal cholesterol to PC-SUV probably was not due to diversion of the sterol to nonlysosomal contaminants in the donor fraction. This explanation is unlikely based on data showing little additional stimulation of cholesterol release when the PC-SUV concentration was raised above 1 mg/ml (Fig. 2), and other data showing no reduction in cholesterol release when incubations were supplemented with unlabeled donor fraction (Results). The mechanism of transfer in the present experiments probably was confined to unmediated diffusion of cholesterol from the lysosomal membrane through the intervening aqueous phase to PC-SUV. Aqueous diffusion is the mechanism by which cholesterol moves between artificial vesicles and between the plasma membrane and a variety of extracellular acceptors (45). In studies of this process in vesicle systems, slow rates of transfer similar to those in the present studies were obtained when the donor vesicles contained a high fraction of sphingomyelin (46). Interestingly, the lysosomal membrane is relatively rich in sphingomyelin in comparison to other cellular membranes (47). Thus, the slow release of cholesterol from lysosomes to PC-SUV may be due to the high sphingomyelin content of the donor membrane and the fact that conditions permitted only unmediated diffusional release of lipids. Although this is a plausible explanation for the present results, it also indicates that the mechanism of transport in intact cells probably is not confined to unmediated aqueous diffusion. One or more other mechanisms seem to be operating to allow efficient delivery of lysosomal cholesterol to the plasma membrane and other sites of cholesterol deposition. This may involve some form of facilitated diffusion, intermembrane contacts, intermembrane vesicle trafficking, or a combination of these mechanisms.

Nature of the transport defect in CHO(2-2) cells

Despite the relatively slow transfer of cholesterol from lysosomes to PC-SUV, it was still possible using the present methods to demonstrate the sterol transport defect in CHO(2-2) cells (Fig. 4). Two conclusions are suggested by this result. First, it indicates that these

methods reproduce at a qualitative level some important aspect of cholesterol transport in intact cells. Thus, it is suggested that lysosomal cholesterol transport in cells involves some form of diffusion, probably facilitated by one or more proteins to make it more efficient than unmediated aqueous diffusion. Whether or not conditions provide for mediation, sterol release from CHO(2-2) lysosomes apparently is less efficient than from normal lysosomes. This leads to the second conclusion, which is that the defect in CHO(2-2) cells appears to reside at least partially in lysosomes. This contrasts with the possibility that lysosomes function normally in these cells and that the lysosomal accumulation of cholesterol is due to a transport defect at a site downstream in an obligatory transport pathway. In these studies we also found that the release of total membrane cholesterol was reduced using the CHO(2-2) donor fraction (Results). This may indicate that the transport defect in these cells is not confined to lysosomes, but affects other membranes or organelles as well. Assuming homology between the defects in CHO(2-2) cells and type C Niemann-Pick disease, this finding may help to explain the observation by Coxey et al. (48) that cholesterol accumulates in both the lysosomes and the trans-Golgi apparatus of type C Niemann-Pick fibroblasts. The genetic relationship between CHO(2-2) cells and type C Niemann-Pick disease is not yet established.

Relevance to atherosclerosis

The cholesterol that accumulates in atherosclerotic plaque is derived largely from LDL, and its tissue deposition is thought to involve the endocytosis and lysosomal degradation of native and oxidatively modified forms of the lipoprotein by cells of the arterial wall (49). In advanced stages of atherosclerosis, there is considerable accumulation of cholesterol and cholesteryl ester in foam-cell lysosomes (50). Thus, the prevention and reversal of atherosclerosis may depend on the efficient transport of lysosomal cholesterol to the plasma membrane, from which it can desorb to extracellular carriers such as high density lipoprotein for transport to the liver. The relevance to atherosclerosis is one of the motivations for developing a better understanding of the transport of lysosomal cholesterol in cells. In the present studies, the data most relevant to atherosclerosis are those addressing the effects of Ca^{2+} ion (Fig. 3). Data from other sources indicate that the intracellular Ca^{2+} concentration in cells of atherosclerotic plaque may approach 1 μM (31), and that cellular Ca^{2+} levels (as modified by pharmacologic agents) are positively associated with metabolic changes indicative of intracellular cholesterol accumulation (51–53). In the present studies, we found that the elevation of Ca^{2+} into the micromolar range lead to enhanced LDL degradation and diminished release of free cholesterol to PC-SUV medium (Fig. 3). The mechanism of these effects is not established, although it has been reported that Ca^{2+} ion can enhance endosome-endosome fusion (30). If the same applies to endosome-lysosome fusion, the enhanced degradation of LDL is reasonable. Regardless of the mechanism by which Ca^{2+} exerts its effects, the present results support the existence of an association between cellular Ca^{2+} levels and the metabolism of cholesterol. The results suggest that elevated Ca^{2+} may lead directly to cholesterol accumulation in lysosomes, and thereby contribute to the progression of atherosclerosis. The cell-free procedures described in this paper may prove valuable for establishing the mechanism by which Ca^{2+} modifies the metabolism of LDL and for exploring the link between cellular Ca^{2+} accumulation and progression of atherosclerosis.

Conclusions

The present studies establish procedures for examining cholesterol transfer from intact functional lysosomes to membrane acceptor particles under cell-free conditions. Using these methods, qualitative reproduction of the transport defect in CHO(2-2) cells was obtained, suggesting that the transport defect in these cells may be localized at least in part to lysosomes. Using phospholipid vesicles as the acceptor, the maximal rate of cholesterol release was slower than occurs in intact cells, suggesting that further refinements are needed for the full reconstitution of the cellular transport of lysosomal cholesterol. Adaptations of these methods should prove useful in the examination of various aspects of lysosome function. The full reconstitution of the transport of lysosomal cholesterol will require the addition of cell-derived acceptors and possibly cytosolic proteins. The present work lays the foundation for this reconstitution.

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