Transport of Cholesterol from the Endoplasmic Reticulum to the Plasma Membrane

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ABSTRACT We have studied the transport of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane in Chinese hamster ovary cells using a cell fractionation assay. We found that transport is dependent on metabolic energy, but that the maintenance of the high differential concentration of cholesterol in the plasma membrane is not an energy-requiring process. We have tested a variety of inhibitors for their effect on cholesterol transport and found that cytochalasin B, colchicine, monensin, cycloheximide, and NH_4Cl did not have any effect. The cholesterol transport process shows a sharp temperature dependence; it ceases at 15°C, whereas cholesterol synthesis continues. When synthesis occurs at 15°C, the newly synthesized cholesterol accumulates in the endoplasmic reticulum and in a low density, lipid-rich vesicle fraction. These results suggest that cholesterol is transported via a vesicular system.

Mammalian cells obtain the cholesterol essential for their growth by two mechanisms: uptake from blood serum in the form of low density lipoprotein–bound cholesterol ester and by *de novo* synthesis. Under conditions of limited exogenous cholesterol, the endogenous cholesterol provides the cell's demand. Little is known about the mechanism by which newly synthesized cholesterol is distributed in the cell. It has been shown that the enzymes involved in hepatic cholesterol biosynthesis are located in the endoplasmic reticulum (1). From the site of synthesis, cholesterol is transported to the various cellular membranes. In nonsecreting, nonsteroidogenic cells, the major destination is the plasma membrane, which is highly enriched with this lipid. Neither the mechanism of transport nor the specificity of this process has been described.

There are several possible transport mechanisms to be considered. One mechanism is a carrier protein–mediated transport. Stimulation of some steps in the biosynthetic pathway between squalene and cholesterol by a sterol carrier protein present in liver cytosol has been demonstrated (2). Cytosol from rat liver causes a slight enhancement of the rate of cholesterol exchange between membranes in vitro (3). Chanderbahn et al. (4) have shown in vitro that sterol carrier protein mediates the transfer of cholesterol from adrenal lipid droplets to mitochondria. There has been no evidence, however, to support a role for this protein in the in vivo transport of newly synthesized cholesterol to the plasma membrane.

Another mechanism that has been considered is differential partitioning of cholesterol in membranes due to different phospholipid composition. This mechanism implies passive diffusion of monomers through the cytoplasm. Wattenberg and Silbert (5) have concluded that differential partitioning cannot account for the large differences in cholesterol distribution found in vivo.

Considering the physical properties of cholesterol, vesicle-mediated transport would seem a likely possibility. De Silva and Siu (6) have reported that newly synthesized phospholipid is transported via lipid-rich vesicles in *Dictyostelium discoidium*. They found this process to be dependent on energy and inhibited by cytoskeleton-disrupting agents. Crivello and Jefcoate (7) found that cholesterol transport to mitochondria in adrenal cells is inhibited by cytoskeleton-disrupting agents.

We have recently reported a direct measurement of the rate of transport of cholesterol and phosphatidylcholine to the plasma membrane (8, 26). The technique involved pulse–chase radiolabeling of cholesterol in vivo followed by rapid isolation of the plasma membrane with cationic beads. Using this method, we demonstrated that at 37°C, cholesterol transport to the plasma membrane proceeds without a time lag, has a half-time of ~10 min, appears to be energy dependent, and ceases at 0°C (8).

In this report, we extend our previous work by using pulse–chase labeling followed by cell fractionation, in addition to using the cationic beads technique. The cell fractionation

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procedure allows us to directly follow the movement of cholesterol from the endoplasmic reticulum to the plasma membrane, as well as identify possible intermediates in the transport process.

MATERIALS AND METHODS

Materials: ^{3}H-Acetate (2.8 Ci/mmol) and 1,2-^{14}C-acetate (55.0 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Cytochrome *c* (horse heart), NADPH, cytochalasin B, colchicine, and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO). Monensin was from Calbiochem-Behring Corp. (La Jolla, CA). Silica gel G plates, 250 μm, were from Analtech, Inc. (Newark, DE). Tissue culture media and fetal calf serum were from Gibco Laboratories (Grand Island, NY).

Cell and Culture Conditions: Chinese hamster ovary cells were grown in suspension culture in a moist 5% CO_2 environment at a density of 0.4–1 × 10^6 cells/ml in alpha minimal Eagle's medium supplemented with 10% lipoprotein-depleted fetal calf serum (9), 0.1 mg/ml of streptomycin, and 100 IU/ml of penicillin.

Prelabeling and Pulse–Chase Radiolabeling of Cells: 16–24 h before the experiment, cells were harvested by centrifugation and resuspended at 4 × 10^5 cells/ml in pyruvate-free medium. At this time, ^{14}C-acetate was added (1 μCi/100 ml suspension), and cells were allowed to grow for 16–24 h. Then cells were harvested, washed once with incubation medium, and resuspended in incubation media at 5–10 × 10^6 cells/ml. The incubation medium was essentially the growth media except that it was pyruvate-free and buffered with 15 mM HEPES pH 7.0 rather than carbonate. After a 10-min preincubation at 37°C (or 15°C when indicated), pulse labeling was initiated by adding ^{3}H-acetate (5.6 μCi/10^6 cells, 10–20 μM acetate), and incubation continued for 4 min (or 1 h at 15°C where indicated). The chase was initiated by addition of acetate (155 mM pH 7.0) to a final concentration of 20 mM. At the indicated times, cells were removed and processed for cholesterol analysis.

Energy Inhibition Experiments: Energy inhibitors were added from a neutral solution of 1 M KF and 0.1 M KCN diluted to 310 mosM. The inhibitor mixture was added to the cell suspension to a final concentration of 20 mM KF and 2 mM KCN.

Cell Fractionation: The cell fractionation procedure was developed by Green et al. (10) and used by others (11). Throughout the procedure, special attention was paid to keeping the solutions and tubes on ice. At the indicated times during the chase, cells were removed, quickly poured into 3 vol of ice cold phosphate-buffered saline (PBS), and held on ice. Cells were spun at 600 *g* for 5 min at 4°C and washed once with 50 ml of cold PBS. The cell pellet was resuspended in 10 ml homogenizing buffer (10 mM Tris-HCl, pH 7.4, 0.3 M sucrose) and spun for 2 min at 1,000 *g*. The pellet was resuspended in the same buffer (1–2 × 10^8 cells/ml), and cells were disrupted using a tight-fitting Dounce homogenizer (20 strokes). The homogenate was spun for 5 min at 600 *g* at 4°C. The resulting postnuclear supernatant solution was spun 5 min at 10,000 *g* at 4°C yielding a mitochondrial pellet and a postmitochondrial supernatant fraction. The two pellets (nuclear pellet and mitochondrial pellet) were washed with 0.3 ml each of homogenizing buffer and spun for 5 min at 10,000 *g*. The wash supernatant fraction was added to the postmitochondrial supernatant fraction. The resulting pooled fraction was diluted 1:1 with a pH 6.8 buffer (0.3 M sucrose, 25 mM HEPES, 25 mM KCl, 2 mM MgAc, pH 6.8). The postmitochondrial supernatant was adjusted to 49% (wt/wt) sucrose with 63.5% (wt/wt) sucrose in Tris-8.0 (1 mM EDTA, 1 mM Tris-HCl, pH 8). The postmitochondrial supernatant fraction from 1–2 × 10^8 cells is loaded per centrifuge tube in a final volume of 5.7–8 ml. This was overlayed with a step gradient of 5 ml each of 38, 35, 31, 27, 23, and 19% (wt/wt) sucrose in Tris-8 buffer. Gradients were centrifuged for 3 h at 4°C in an SW-27 rotor at 24,000 rpm, and fractions were collected by puncturing the bottom of the tube. Aliquots of each fraction were counted in a scintillation counter, and appropriate fractions were pooled and diluted to <10% sucrose using Tris-8 buffer. After a 1-h centrifugation of the pooled fractions at 10^5 *g* at 4°C, the membrane pellets were resuspended in a small volume and analyzed.

Lipid Analysis: The analysis of cellular lipids was essentially as described by DeGrella and Simoni (8). After lipid extraction (12), samples were separated in two thin layer chromatography (TLC)[1] systems in series: the first TLC system used hexane/ethyl ether/acetic acid (80:20:2); the cholesterol spot of the first system was extracted twice with 2.5 ml ethyl ether/methanol (4:1) and spotted on reverse-phase TLC plates (Silica gel G prerun with 5% Squibb mineral oil [Squibb Corp., Princeton, NJ] in petroleum ether). Plates were developed in methanol/water (9:1). The cholesterol spots were visualized by H_2SO_4 charring. Analysis of these samples by high performance liquid chromatography has shown >80% of the ^{3}H-cpm were associated with cholesterol (8), and the ^{3}H/^{14}C values obtained by TLC and high performance liquid chromatography were in good agreement. For those experiments in which only the long time ^{14}C-acetate prelabeling was involved, [^{14}C]cholesterol was obtained from the first TLC plate where the spot was pure. ^{14}C-Phospholipid was obtained from the spot at the origin of the first TLC plate. To verify that [^{14}C]cholesterol values represent cholesterol mass, we performed the following experiment: cell homogenates were fractionated on sucrose gradients as described above and the cholesterol isolated from various fractions as well as from whole cells. Cholesterol mass (14) was determined as well as [^{14}C]cholesterol. There was a good correlation between those values (428 ± 54 cpm/μg cholesterol in six different samples), indicating that the prelabeling procedure yields cellular cholesterol of constant specific activity.

Other Methods: Plasma membrane isolation on beads was done as described by DeGrella and Simoni (8) using cell suspensions at a concentration of 10^7/ml. Protein was determined by the method of Lowry et al. (13). NADPH-cytochrome *c* reductase was assayed according to Phillips and Langdon (15).

Data Analysis: The lipid analysis procedure described above gives ^{3}H/^{14}C ratios in cholesterol. The [^{3}H]cholesterol represents the newly synthesized cholesterol, and [^{14}C]cholesterol represents cholesterol mass. [^{3}H]/[^{14}C]Cholesterol thus indicates the fraction's enrichment in newly synthesized cholesterol. The results of the cell fractionation experiments (Figs. 2, 4, and 7 and Table II) are expressed as relative [^{3}H]/[^{14}C]cholesterol (the [^{3}H]/[^{14}C]cholesterol in the membrane fractions divided by [^{3}H]/[^{14}C]cholesterol in the postmitochondrial supernatant). The use of relative values is required since the chase of radioactive acetate is not immediately effective, which causes the [^{3}H]/[^{14}C]cholesterol in whole cells to increase throughout the chase (8). Our attempts to improve the effectiveness of the chase by modifying chase conditions proved unsuccessful. The data from the bead/plasma membrane isolation procedures (Figs. 5 and 6) is presented as the percent of total cell [^{3}H]cholesterol found in the plasma membrane fraction as described perviously (8).

RESULTS

Our previous study, using a bead/plasma membrane isolation procedure, demonstrated that cholesterol transport to the plasma membrane could be stopped at 0°C (8). This means that cholesterol can be trapped at an early compartment before reaching the plasma membrane. It also means that cholesterol exchange between membranes during sample processing could probably be prevented if samples were kept cold. It thus seemed worthwhile to attempt a more traditional cell fractionation procedure to analyze the intermediate steps of the transport process.

The fractionation procedure avoids pelleting of the membranes which would bring them in close contact and possibly facilitate cholesterol exchange. In order to have a convenient measure of cholesterol mass and an internal standard, cells were routinely prelabeled with ^{14}C-acetate. As described in Materials and Methods, [^{14}C]cholesterol accurately represents cholesterol mass. Thus, the ratio [^{3}H]/[^{14}C]cholesterol in a given fraction represents the relative enrichment in newly synthesized cholesterol.

Characterization of Subcellular Fractions

Since we have used a well-established and well-characterized procedure for subcellular fractionation of these cells (10, 11), we have only verified that the fractionation has worked as expected. The characterization of the membrane fractions derived from the sucrose gradient fractionation is presented in Fig. 1. The heaviest membrane fraction has a very low cholesterol content and contains most of the NADPH-cytochrome *c* reductase activity. This is our endoplasmic reticulum fraction. The bulk of cholesterol, expressed either as cholesterol/protein or cholesterol/phospholipid, is contained in a lighter fraction (d = 1.138–1.168) that we consider to be

[1] *Abbreviations used in this paper:* LF, light fraction; TLC, thin layer chromatography.

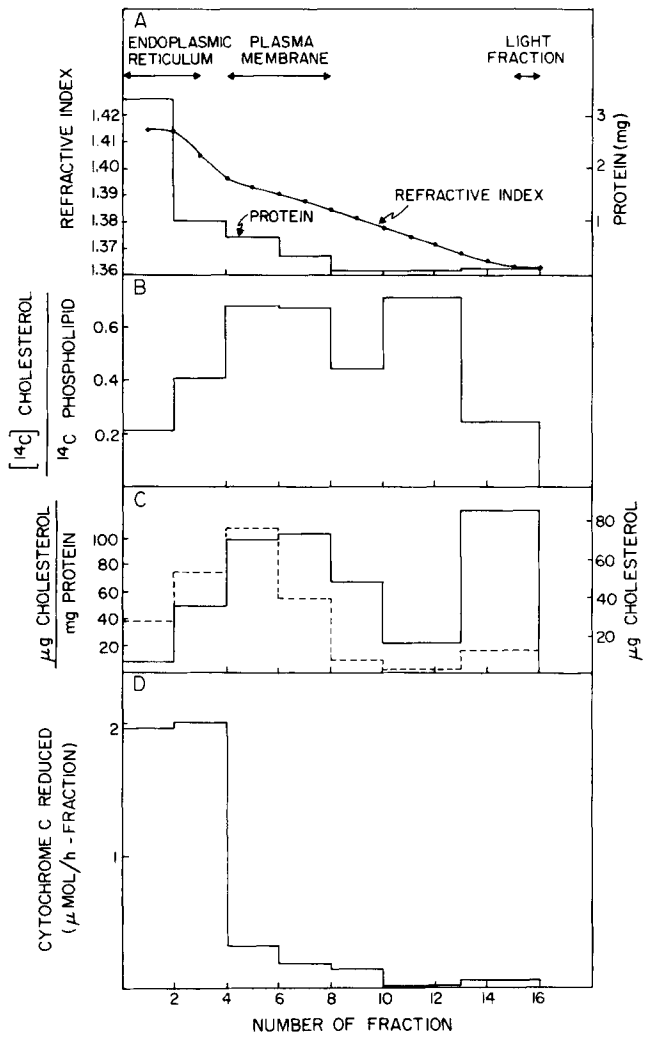

FIGURE 1 Characterization of subcellular fractions. Cells were prelabeled with ^{14}C-acetate and fractionated as described in Materials and Methods. All the solutions contained 2 × 10^{-4} M phenylmethylsulphonyl fluoride. (*A*) The refractive index profile of the gradient (●) and the protein distribution in the washed membrane fractions (*bars*). (*B*) Cholesterol/phospholipid ratio in the washed membrane fractions. (*C*) Cholesterol/protein ratio in the washed membrane fractions. (*D*) NADPH-cytochrome-c reductase activity in washed membrane fractions.

the plasma membrane fraction. This fraction has low NADPH-cytochrome *c* reductase activity. The fractions from the top of the gradient labeled light fraction will be discussed later. We have also indicated the cholesterol/phospholipid ratio (Fig. 1 *B*), which also characterizes the plasma membrane fraction. The peak of cholesterol/phospholipid seen in fractions 10–14 may represent Golgi vesicles. This would be in accordance with the characterization of others.

Kinetics of Cholesterol Transport

The movement of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane was followed at 37°C using a 4-min ^{3}H-acetate pulse. The change in [^{3}H]/[^{14}C]cholesterol values in the plasma membrane and endoplasmic reticulum fractions obtained by cell fractionation is shown in Fig. 2. The results are presented as the ratio of [^{3}H]/[^{14}C]cholesterol in the fractions divided by that of the

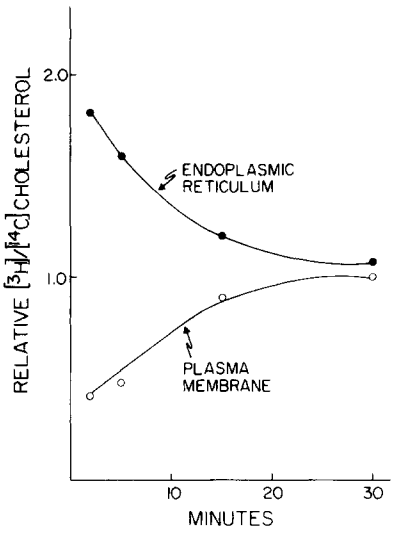

FIGURE 2 Kinetics of cholesterol transport. Cells were prelabeled with ^{14}C-acetate, pulsed with ^{3}H-acetate for 4 min at 37°C, and chased for the times indicated. After cell fractionation, lipid was extracted and [^{3}H]/[^{14}C]cholesterol determined in the endoplasmic reticulum and plasma membrane fractions.

unfractionated postmitochondrial supernatant, as described in Materials and Methods. At the earliest time point after the chase, the [^{3}H]/[^{14}C]cholesterol in the endoplasmic reticulum is ~4.5-fold higher than that in the plasma membrane indicating that, at this time, newly synthesized cholesterol is still primarily in the endoplasmic reticulum. With chase time increasing up to 30 min, this ratio decreases for the endoplasmic reticulum while it increases for the plasma membrane. The distribution of [^{14}C]cholesterol on the gradient remains unchanged at all times. The change in ^{3}H/^{14}C values reflects redistribution of [^{3}H]cholesterol only as it leaves the endoplasmic reticulum and concomitantly appears in the plasma membrane. At 2 min of chase, 40–50% of the [^{3}H]-cholesterol is in the endoplasmic reticulum, dropping to 10% at 30 min. A half-time of ~10 min is obtained from these curves, which is in good agreement with the rate determined previously using the bead technique (8).

When the transport process is complete (~30 min), the [^{3}H]/[^{14}C]cholesterol ratios for both membrane fractions reach the same value. This indicates that [^{3}H]cholesterol has reached its equilibrium distribution within the cell. This is because ^{14}C-acetate labeling over 16–24 h results in a constant specific activity in cholesterol throughout the cell. When the [^{3}H]cholesterol translocation is complete, it will achieve the same distribution pattern as did [^{14}C]cholesterol. We found the use of an internal standard essential in these studies since each time point in an experiment represents a separate sucrose gradient, and losses during processing of gradients and lengthy lipid analyses are inevitable. Expressing the 30-min values in Fig. 2 in counts per minute of [^{3}H]cholesterol per milligram of protein would give the values 3,926 for endoplasmic reticulum and 70,000 for plasma membranes, reflecting the expected plasma membrane enrichment for cholesterol.

Dependence of Cholesterol Transport on Metabolic Energy

We have previously reported that energy poisons prevent

the arrival of newly synthesized cholesterol at the plasma membrane (8). It is reasonable that there be an energy requirement for the process since cholesterol is present in the plasma membrane at much higher concentrations than in the other cellular membranes. We have examined this point in more detail using the cell fractionation procedure to determine which step in the process is energy dependent. When studying the effect of any drug on the rate of cholesterol transport, two requirements should be met. (*a*) Under the experimental conditions used, the rate at which the drug affects the cell must be fast compared with the transport process itself. A drug that also has an effect on cholesterol synthesis, an energy-dependent process, can be added only at the end of the pulse and must act before significant transport has occurred. (*b*) The drug effect should be reversible. A variety of energy inhibitors were screened for these criteria by monitoring their effect on the rate of total lipid synthesis and recovery of the synthesis rate after their removal. In Fig. 3, it is shown that when cells are incubated with 2 mM KCN and 20 mM KF at 37°C, lipid synthesis is stopped completely in <3 min, and the process is rapidly and completely reversible once the inhibitors are removed.

The effect of KCN and KF on the rate of cholesterol transport was assayed using the general pulse-chase protocol and cell fractionation described above, and the results are presented in Fig. 4. The inhibitors were added after the 4-min pulse and were present throughout the 30-min chase. The newly synthesized cholesterol remains primarily in the endoplasmic reticulum but there is significant increase of [^{3}H]-cholesterol in the plasma membrane. Upon removal of the poisons and re-incubation of the cells for an additional 30 min at 37°C in fresh medium, the ^{3}H/^{14}C ratio in the endoplasmic reticulum drops and that in the plasma membrane increases, indicating that transport is resumed as the energy is restored. We conclude from this experiment that the movement of cholesterol from the endoplasmic reticulum to the plasma membrane is energy dependent, and energy depletion results in the cholesterol being unable to leave the endoplasmic reticulum. The apparent continued arrival of some cholesterol at the plasma membrane during energy depletion may indicate that the newly synthesized cholesterol that has already left the endoplasmic reticulum may be able to continue to the plasma membrane in an energy-independent step. This point requires further study.

Effect of Energy Poisons on Intracellular Cholesterol Distribution

It was of interest to find out whether the distribution of cholesterol in the cell changes when the energy inhibitors are added, i.e., whether the maintenance of a cholesterol gradient in the cell is an energy-dependent process. For this purpose the cholesterol/phospholipid ratio in the membrane fractions was determined. Cells labeled with ^{14}C-acetate for 24 h were divided into two samples, and to one sample, KCN/KF was added. Both samples were incubated for 30 min at 37°C, cooled to 0°C, and fractionated. The cholesterol/phospholipid values were derived from the ^{14}C-cpm of the relevant spots on the first TLC plate. The results of this experiment, summarized in Table I, indicate that the cholesterol/phospholipid ratio of the plasma membrane and endoplasmic reticulum are not altered by addition of energy inhibitors. Thus, it appears that although the transport process is energy dependent, once the cholesterol gradient is formed energy is not required for its maintenance.

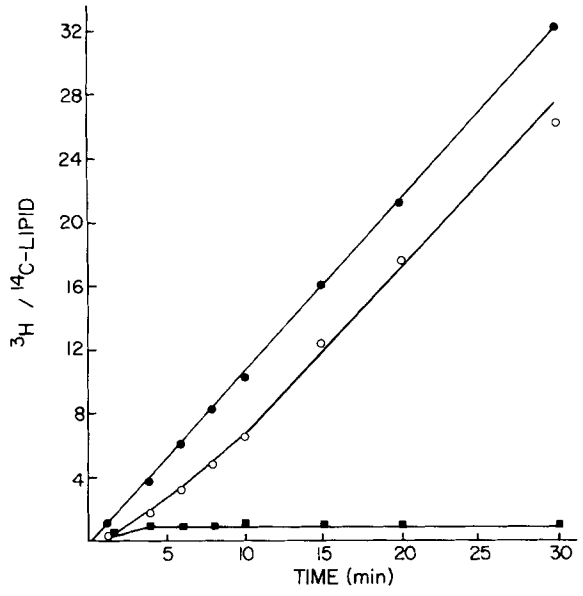

FIGURE 3 Effect of energy inhibitors on lipid synthesis. Cells, 1 × 10^8, were prelabeled with ^{14}C-acetate, then incubated at 37°C with ^{3}H-acetate for the times indicated as in Materials and Methods. Aliquots were removed at various times and were lipid extracted and analyzed without addition of inhibitors (●). As soon as possible before the ^{3}H-acetate, 2 mM KCN, 20 mM KF were added (see Materials and Methods) and samples processed as above (■). Energy inhibitors were added for 30 min at 37°C, then inhibitors were removed by washing. Cells were resuspended in fresh incubation medium and were labeled with ^{3}H-acetate initiated as above (○).

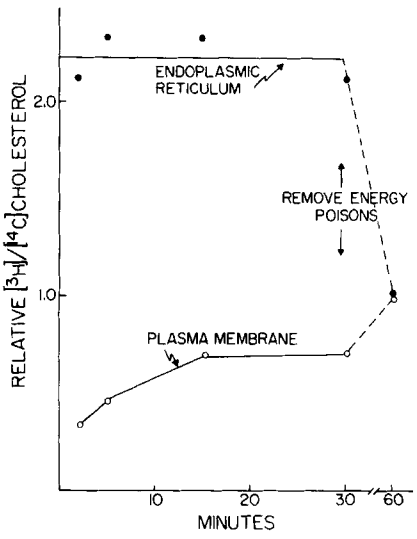

FIGURE 4 Inhibition of cholesterol transport by energy poisons. The same protocol as in Fig. 2 had been used except that energy poisons were added at the end of the 4-min pulse, as described in Materials and Methods. [^{3}H]/[^{14}C]Cholesterol was analyzed in the endoplasmic reticulum (●) and the plasma membrane (○) for the duration of the chase. The last time point represents the recovery of transport after the energy arrest: after energy-inhibition for 30 min, cells were washed three times to remove the poisons and resuspended in fresh incubation media (see Materials and Methods) for 30 min at 37°C. Then cells were fractionated and membrane-lipid analyzed.

Effect of Various Inhibitors on Cholesterol Transport

To gain more insight into the mechanism of cholesterol transport, we tested the effect of various inhibitors. To rapidly and easily screen several drugs, we used the bead/plasma membrane isolation procedure for this study rather than the cell fractionation procedure. Inhibitors that affect cholesterol synthesis have to be added after the ^{3}H-acetate pulse. This was the case for cytochalasin B and colchicine. That these two inhibitors were exerting an effect on cell morphology was verified on cells that have been grown attached to plates rather than in suspension. When 50 μM cytochalasin B was added to the culture, cell rounding was pronounced in <5 min. The effect of 50 μM colchicine was less noticeable. From the data in Fig. 5, it is obvious that no decrease in the transport occurs with any of the inhibitors tested. Thus, cytochalasin B, colchicine, monensin, NH_4Cl, and cycloheximide do not affect cholesterol transport under the conditions tested.

TABLE I. *Effect of Energy Poisons on the Cellular Distribution of Cholesterol*

	Cholesterol/phospholipid	
	No poisons	With poisons
Plasma membrane	0.46 ± 0	0.41 ± 0.03
Endoplasmic reticulum	0.22 ± 0.01	0.18 ± 0.04

Cells were prelabeled with ^{14}C-acetate, then divided into two cultures. To one sample, energy poisons were added (see Materials and Methods) and both samples incubated for 30 min at 37°C. Cells were cooled, harvested, and fractionated, and the lipid of the cellular fractions was analyzed. Results are expressed as [^{14}C]cholesterol/^{14}C-phospholipid in each fraction and show the average and standard deviation of three experiments.

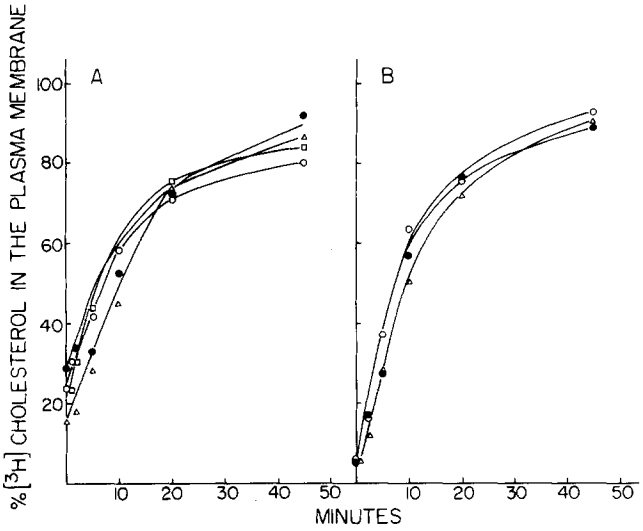

FIGURE 5 Effect of cycloheximide, NH_4Cl, monensin, cytochalasin, and colchicine on cholesterol transport. Cells were prelabeled with ^{14}C-acetate, pulsed with ^{3}H-acetate, chased with acetate, and plasma membranes were isolated by the bead technique at various times during the chase. (*A*) 10 μM monensin was added 1 h before the pulse (△); 20 μM cycloheximide was added 2 h before the pulse (●); 10 mM NH_4Cl was added 30 min before the pulse (○); the control sample was preincubated with 0.1% ethanol, which was used to add the monensin (□). (*B*) 50 μM cytochalasin B (●) or colchicine (△) were added with the chase; the control contained 0.1% ethanol and 1% dimethyl sulfoxide which were used to apply the drugs (○).

Temperature Dependence of Cholesterol Transport

Since it has been reported that some intracellular vesicular transport systems show a sharp temperature dependence (15–18), we decided to examine the temperature dependency of cholesterol transport. Using the bead/plasma membrane isolation procedure, we examined kinetics of arrival of cholesterol at the plasma membrane at 35°, 25° and 15°C, as shown in Fig. 6. In all three cases, the pulse of ^{3}H-acetate is conducted at 37°C and the temperature than lowered as fast as possible (50 s to reach 15°C) for the chase. While the transport rate slows roughly threefold when the temperature is lowered from 35° to 25°C (half times of ~9 min and 26.5 min, respectively), the transport is stopped or is extremely slow when the tem-

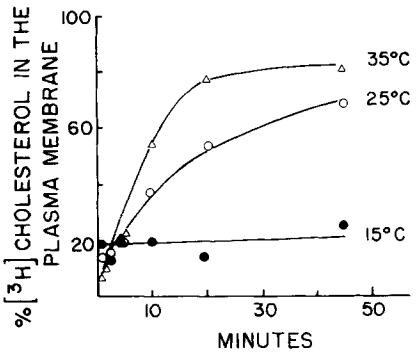

FIGURE 6 Temperature dependence of cholesterol transport. Cells were prelabeled with ^{14}C-acetate and pulsed for 4 min at 37°C. As the chase was initiated and the temperature was quickly lowered in an ice water bath to the desired temperature (50 s to reach 15°C), that was maintained throughout the chase. Samples were withdrawn at the indicated times during the chase and plasma membrane isolated on beads. △, 35°C; ○, 25°C; ●, 15°C.

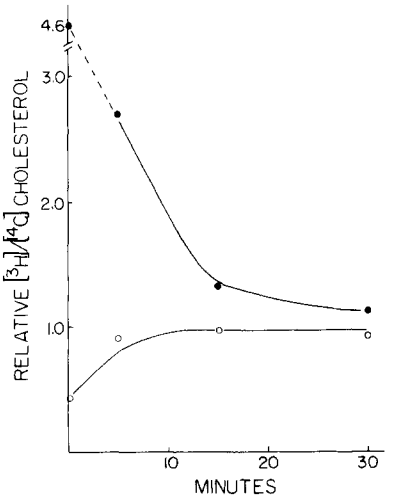

FIGURE 7 Arrest of cholesterol transport at 15°C and recovery at 37°C. Cells were prelabeled with ^{14}C-acetate, then labeled with ^{3}H-acetate for 1 h at 15°C followed by a 30-min chase at 15°C. Cells were then quickly warmed to 37°C, samples withdrawn at the indicated times, cells fractionated on sucrose gradients, and the lipid of the endoplasmic reticulum (●) and plasma membrane fractions (○) extracted and analyzed.

perature is dropped to 15°C.

To examine this transport arrest at 15°C in more detail, the cell fractionation assay has been used to find out in which membrane(s) cholesterol accumulates at 15°C. The results are shown in Fig. 7. Cells were pulse-labeled with ^{3}H-acetate at 15°C for 1 h followed by a 30-min chase also at 15°C. The absolute synthesis rate is about fourfold lower at 15°C compared with that at 37°C. When the cells are fractionated, it is apparent that the transport arrest at 15°C results in the accumulation of newly synthesized cholesterol in the endoplasmic reticulum fraction, resulting in a 10-fold higher ^{3}H/^{14}C-ratio in this fraction compared with that in the plasma membrane. These values are the zero time points in Fig. 7. Cells were then rapidly warmed to 37°C, and the distribution of [^{3}H]-cholesterol in membrane fractions was observed for 30 min as indicated in Fig. 7. Warming the cells to 37°C causes the cholesterol to move from the endoplasmic reticulum to the plasma membrane in a process similar to the normal rate described in Fig. 2. This is reflected by a decrease of [^{3}H]/[^{14}C]cholesterol in the endoplasmic reticulum accompanied by an increase in the plasma membrane.

Accumulation of Newly Synthesized Cholesterol in a Low Density Fraction

When cholesterol synthesis from ^{3}H-acetate was conducted for 1 h at 15°C and the cell homogenates fractionated on sucrose gradients as described above, it was observed that ^{3}H-lipid appeared at the top of the sucrose gradient ($d < 1.087$) (Fig. 8*A*). This is not observed when the ^{3}H-acetate pulse is conducted at 37°C (data not shown, but identical to that in Fig. 8*B*) or when synthesis at 15°C was followed by a 30-min warming at 37°C (Fig. 8*B*). Isolation and analysis of the lipid in this light fraction (LF) from the sucrose gradient reveals the following in Table II: the cholesterol in that fraction has a high ^{3}H/^{14}C ratio indicating that this fraction is enriched with newly synthesized cholesterol. After 30 min at 37°C, the [^{3}H]/[^{14}C]cholesterol reaches the same value as that of the other membranes, namely, its relative enrichment with newly synthesized cholesterol is no longer observed, indicating that the accumulated [^{3}H]cholesterol goes to the plasma membrane. These results provide preliminary evidence that this fraction may represent an intermediate in the transport process. We have analyzed the properties of this gradient fraction. If the sucrose concentration in the LF is diluted and the solution centrifuged at 100,000 *g* for 1 h, 90% of the [^{3}H]-cholesterol is found in the pellet fraction. This material contains 4–8% of the cholesterol mass compared with that in the plasma membrane. It has a high cholesterol/protein ratio characteristic of plasma membranes but a cholesterol/phospholipid ratio resembling that of the endoplasmic reticulum fraction (Fig. 1). Electron miroscopic examination of the LF indicates smooth membrane vesicles (data not shown).

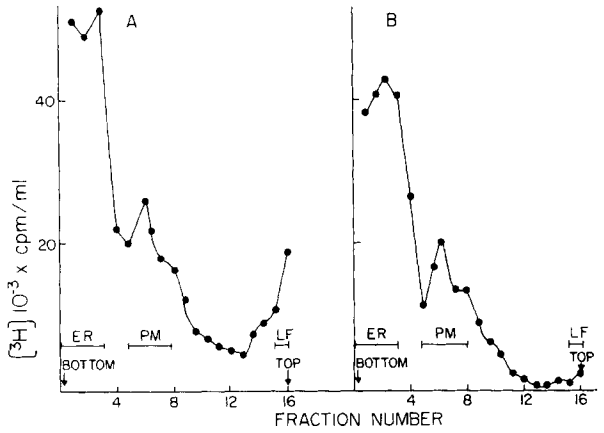

FIGURE 8 Accumulation of newly synthesized lipid in a low-density fraction. Cells were prelabeled with ^{14}C-acetate, labeled with ^{3}H-acetate for 1 h at 15°C, and chased for 30 min at 15°C. Half of the cells were cooled to 0°C (*A*) and half warmed to 37°C for 30 min (*B*). Both samples were fractionated, and aliquots of the gradient fractions were counted for ^{3}H.

TABLE II. *Newly Synthesized Cholesterol in a Low Density Vesicle Fraction*

	Relative [^{3}H]/[^{14}C]cholesterol		
	15°C	15°/37°C	37°C
Endoplasmic reticulum fraction			
Exp. 1	2.9	1.0	1.0
Exp. 2	4.6	1.1	n.d.
Plasma membrane fraction			
Exp. 1	0.5	1.0	1.0
Exp. 2	0.4	0.9	n.d.
Light fraction			
Exp. 1	1.8	0.9	1.0
Exp. 2	2.	0.9	n.d.

Cells were prelabeled with ^{14}C-acetate, then split into three samples. Cell samples were either labeled with ^{3}H-acetate for 1 h at 15°C followed by a 30-min chase at 15°C (first column), or labeled as above, then warmed to 37°C for 45 min (second column). The third sample was pulsed at 37°C for 4 min and chased at 37°C for 45 min (third column). Samples were then fractionated and the lipid analyzed. Values are given as relative [^{3}H]/[^{14}C]-cholesterol (Materials and Methods). Results of two separate experiments are presented. n.d., not determined.

DISCUSSION

In this study, we have extended our previous examination of intracellular cholesterol transport by using a cell fractionation procedure in addition to the bead/plasma membrane isolation procedure (8). In this current work, we have confirmed the half-time of ~10 min for cholesterol transport determined previously (8). We also demonstrate that in the absence of metabolic energy, newly synthesized cholesterol cannot leave the endoplasmic reticulum. The effect is reversible; when energy poisons are removed, transport resumes. It is obvious from the data presented in Fig. 4 that the newly synthesized cholesterol in the endoplasmic reticulum does not decrease as it does in the control experiment (Fig. 2). However, as can also be seen in Fig. 4, there is some increase in the appearance of newly synthesized cholesterol in the plasma membrane even in the presence of energy poisons at the early time points. One possible explanation is that energy depletion affects cholesterol when it leaves the endoplasmic reticulum, but does not affect the later steps needed to reach the plasma membrane. The dependence on metabolic energy argues against passive diffusion coupled to differential cholesterol partitioning (19, 20) as a mechanism for cholesterol transport. Whereas energy is required to transport cholesterol, energy is not required to maintain the high cholesterol concentration in the plasma membrane. This result is in agreement with the

observation of Lange and Ramos (21) that cholesterol in the plasma membrane is not in rapid equilibrium with internal membranes. This has important implications with regard to the fate of cholesterol added exogenously to cells.

Cytochalasin B, colchicine, monensin, cyclohexamide, and NH_4Cl do not affect cholesterol transport. There are varied data in the literature about the effect of cytoskeleton-disrupting agents on lipid transport processes. DeSilva and Siu (6) reported sensitivity to colchicine of phospholipid transfer in *Dictyostelium*, and Crivello and Jefcoate (7) found that cholesterol transport to mitochondria in adrenal cells is sensitive to cytochalasin B and vinblastine. Sleight and Pagano (22) studying phosphatidylethanolamine transport to the plasma membrane in fibroblasts reported lack of sensitivity to cytochalasin or colchicine. Monensin has been shown to inhibit intracellular transport of membrane and secretory proteins (23, 24) as well as endocytotic processes (25). We found no effect of monensin on cholesterol transport.

There is a dramatic temperature dependency of the cholesterol transport process. Below 15°C, transport is effectively stopped. Similar temperature-dependence profiles have been shown in the case of endocytotic processes where the transported proteins or viruses are accumulated in prelysosomal compartments (16, 17). It has also been shown that hemagglutinin transport to the cell surface is prevented at 20°C, even though terminal glycosylation continues (18). In our studies, cells were kept at 15°C for up to 90 min while cholesterol synthesis continued and little or no transport to the plasma membrane occurred (Fig. 7). This observation allowed us to separate cholesterol transport from synthesis. With fourfold more synthesis at 15°C/60 min than at 37°C/4 min, little or no transport took place. This does not, of course, rule out the reciprocal possibility that transport requires synthesis. In the studies with various inhibitors (see Fig. 5), it can be tentatively concluded that the transport rate is independent of synthesis rate. Some of these agents alter cholesterol synthesis rates such as cholchicine, which stimulates about twofold, and cytocholasin B, which inhibits ~50%. This is not apparent in Fig. 5 since the data treatment normalizes for differences in synthesis rates. It is clear, however, that over this range of synthesis the transport rates do not vary.

When cholesterol synthesis and transport are monitored at 15°C, most of the newly synthesized cholesterol remains in the endoplasmic reticulum but some is found in a very low density fraction (Fig. 8*A*, Table II). When cells are warmed to 37°C, the enrichment of newly synthesized cholesterol in this fraction is no longer observed (Fig. 8*B*, Table II), and we detect that the [^{3}H]cholesterol in this fraction moves to the plasma membrane fraction. It is unlikely that the [^{3}H]cholesterol that is detected in the LF is due to contamination with endoplasmic reticulum since we would expect to see an enrichment of newly synthesized cholesterol with the standard 4-min pulse at 37°C, and this is not observed (data not shown but identical to that in Fig. 8*B*). The characterization of this LF is preliminary, and further work is required to assess the purity of these vesicles. This has been extremely difficult so far because there is very little material present in this fraction. It is clear, however, there are lipid-rich vesicles in which newly synthesized cholesterol accumulates at 15°C. The disappearance of this newly synthesized cholesterol from this fraction suggests that these vesicles are possibly endoplasmic reticulum-derived intermediates in the transport of cholesterol to the plasma membrane.

The measurement of intracellular transport of cholesterol in amoeba has been reported by Mills et al. (27) who found that at 25°C, there is a 30-min lag before cholesterol appears in the plasma membrane. Lange and Ramos (21) have reported that endogenously synthesized cholesterol, in intact fibroblasts, is resistanct to cholesterol oxidase action for several hours, suggesting that it takes ~12 h for 50% of the endogenously synthesized cholesterol to reach the surface membrane. More recently, Lange and Matthies (28) have reported that the half time for cholesterol transport to the surface in fibroblasts is ~1–2 h, again using a cholesterol oxidase assay for surface accessibility. Assuming their 1–2-h value, this transport rate is still significantly slower than is the half time of 10–15 min that we have reported using both the bead assay and the cell fractionation assay. Assuming both rates are accurate, the difference obtained may reflect the different assays used. In order for cholesterol to be oxidized by cholesterol oxidase from the outside of the cell, it must be in the plasma membrane phospholipid bilayer. In contrast, in order for cholesterol to be detected in the plasma membrane fractions using our bead or cell fractionation assay, the cholesterol need only be associated with the plasma membranes. If cholesterol is transported from the endoplasmic reticulum to the plasma membrane in vesicles, then our bead and cell fractionation assays would detect the arrival of the vesicles, whereas the cholesterol oxidase assay would require that vesicle fusion occur. If the fusion step is rate limiting, it could explain the differences in the rates determined with the different assays.

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