Transfer of Cholesterol from Its Site of Synthesis to the Plasma Membrane*

Yvonne Lange and Heinrich J. G. Matthies

From the Departments of Pathology and Biochemistry, Rush Medical College, Chicago, Illinois 60612

We have followed the transfer of newly synthesized cholesterol to the plasma membrane in cultured fibroblasts using cholesterol oxidase as a probe. Since the enzyme has access only to the plasma membrane in intact cells, it permits the discrimination of cell-surface and endogenous cholesterol. Cholesterol synthesized from radiolabeled acetate was transferred to the plasma membrane in a strictly first order fashion with a half-time of 1-2 hours at 37°C. The rate of transfer was similar in rapidly growing and confluent cells and was not affected by preincubating the cells in lipoprotein-deficient serum which greatly stimulated cholesterol synthesis. We used equilibrium density gradient centrifugation of homogenates from cholesterol oxidase-treated cells to examine further the distribution of newly synthesized cholesterol between cellular pools. We identified membrane fractions enriched in newly synthesized cholesterol yet inaccessible to cholesterol oxidase. The cholesterol in these membranes eventually moved to the plasma membrane. The movement of exogenous radiocholesterol from the plasma membrane to the cell interior also was examined by this method. No detectable transfer was observed over several hours, during which time endogenous cholesterol moved to the plasma membrane. We conclude that the transfer of newly synthesized cholesterol to the plasma membrane is a vectorial process and is not mediated by a simple diffusional equilibrium.

While there is considerable information concerning the movement of newly synthesized proteins to the plasma membrane (1, 2), the pathway by which lipid is incorporated into the plasma membrane is not well understood. The transfer of newly synthesized phospholipid to the plasma membrane has been examined recently in Dictyostelium discoideum (3) and cultured Chinese hamster fibroblasts (4); however, different mechanisms appear to be involved in these two systems. More than 90% of the cholesterol in human fibroblasts and Chinese hamster ovary cells is localized in the plasma membrane (3), but the mechanism of this segregation is unknown. Studies of the rate of transfer of cholesterol from its site of synthesis to the plasma membrane have given conflicting results. In a study of radiocholesterol movement between human skin fibroblasts and sonicated liposomes, Poznansky and Czekanski (6) found no movement of endogenously synthesized cholesterol to the cell surface in the course of several hours. However, DeGrella and Simoni (7) showed that the movement of newly synthesized cholesterol to the plasma membrane in Chinese hamster ovary cells occurred with a half-time of approximately 10 minutes.

We recently have developed a technique which permits plasma membrane and endogenous cholesterol pools to be distinguished in intact cells (5). The method is based on the use of the enzyme cholesterol oxidase, which attacks surface cholesterol under certain conditions, leaving internal cholesterol pools untouched. Because of the rapid equilibration of cholesterol across the plasma membrane (5, 8), the entire pool of cell surface cholesterol can only be quantified, but can be physically separated, as cholestenone, from the internal cholesterol. This finding provides an approach to the measurement of the rate at which newly synthesized cholesterol moves to the plasma membrane. Our strategy was to incubate cells in culture with radiolabeled acetate and use cholesterol oxidase to follow the distribution of labeled cholesterol between plasma membrane and internal pools.

EXPERIMENTAL PROCEDURES

Materials—[^H]Acetic acid (sodium salt, 90.0 mCi/mmol), [4C]acetic acid (sodium salt, 56.0 mCi/mmol), [7-3H]cholesterol (34.6 Ci/mmol), and [4-14C]cholesterol (60 mCi/mmol) were purchased from New England Nuclear. The radiolabeled cholesterol was purified to >99% by thin-layer chromatography prior to use. We checked periodically that the radiolabeled cholesterol stocks were completely oxidizable by cholesterol oxidase in detergent solution. [4C]Cholestenone was prepared from [4C]cholesterol using cholesterol oxidase. The product was purified by thin-layer chromatography. Cholesterol (>90% pure) and cholestenone were obtained from NuCheck Prep (Elysian, MN) and Steraloids Inc. (Wilton, NH), respectively. Tissue culture media and fetal calf serum were obtained from Gibco Laboratories (Grand Island, NY) and Hyclone (Logan, UT), respectively. Cholesterol oxidase (EC 1.1.3.6; Brevibacterium sp.) was used as obtained from Beckman Instruments. Analysis of the enzyme by gel electrophoresis (9) showed a single band. Thin layer chromatography plates were obtained from Whatman.

Cell Culture—Cultured human fibroblasts were derived from the foreskin of healthy newborns. Cells were grown in monolayer and used between the 5th and 11th passages. Cultures were maintained at 37°C in a humidified incubator (5% CO2) in 75-cm² or 175-cm² flasks containing Dulbecco’s modified Eagle’s minimal medium plus 10% fetal calf serum. Twenty-four to forty-eight hours prior to the initiation of most experiments, the medium was removed from the flasks, the monolayers of cells were rinsed twice with serum-free medium, and fresh medium containing 5% (v/v) fetal calf lipoprotein-deficient serum prepared as described (10) was added.

Chinese hamster ovary cells were obtained from the American Type Culture Collection and grown in 75-cm² flasks using the conditions described above for fibroblasts.

Labeling Cellular Lipids—Radiolabeled acetate (0.075–0.25 mCi) was added in ethanol to the 7-ml or 15-ml medium of each flask and incubated at 37°C for various times. The medium then was removed and in some experiments the label was chased by the addition of fresh medium containing 10 mM Na acetate for varied intervals. The cells then were washed twice in the flask with phosphate-buffered saline, 15 mM NaCl, 5 mM NaF, (pH 7.5), 0.25% trypsin, 0.03% EDTA in phosphate-buffered saline prewarmed to 37°C were added and the
flask was incubated for 5 min at 37 °C to release the cells. After removal from the flask, the cells were washed twice more in phosphate-buffered saline at 0 °C prior to cholesterol oxidase treatment.

Treatment of Cells with Cholesterol Oxidase—Washed cells were suspended in approximately 10 volumes of phosphate-buffered saline and duplicate aliquots were taken for the determination of protein. The remainder was made 1% in glutaraldehyde and incubated for 10 min on ice. The cells were washed twice in 0.5 mM NaPi, 310 mM sucrose (pH 7.5) and resuspended in this buffer at 10 °C. This pretreatment greatly increases the sensitivity of membrane cholesterol to this enzyme (11). Cholesterol oxidase was added to 8–10 IU/ml and the cells were incubated for 45 min at 10 °C. The mixture was plated on ice, brought to isotonicity by the addition of 1.5 mM NaCl, 5 mM NaPi, and extracted with 5 volumes of chloroform:methanol (2:1, v/v). 'H-labeled cholesterol and cholestenone were added with the organic solvent to permit determination of recovery which averaged 85%.

Density Gradient Fractionation—Radiolabeled cells (approximately 4–6 mg of protein) were washed twice in 0.5 mM NaPi, 310 mM sucrose (pH 7.5) and resuspended in this buffer at 0 °C. The suspension was brought to 1 ml in 0.25 M sucrose, 5 mM NaPi, and the cells were homogenized in a glass-glass coaxial pestle homogenizer using 25–35 strokes. Homogenates were spun for 5 min at 800 x g to remove unbroken cells and nuclei and the resulting supernatants were layered on linear gradients of 19.5%–62% sucrose (w/v) in 50 mM NaPi (pH 8). The gradients were spun in a Beckman SW40 rotor for a minimum of 10 X g, min at 2 °C. Gradients were collected in equal volume fractions from the bottom of the tube. Aliquots of each fraction were taken for refractive index determination and the remainder was extracted for the analysis of radioactivity.

Determination of Radioactivity in Cholesterol and Cholestenone—Lipid extracts were spotted together with standards on Silica Gel G plates. The plates were developed in chloroform:methanol (100:2, v/v) and visualized with I, vapor. The appropriate region of the plate was scraped and the material was eluted twice with 2 ml of chloroform:methanol (2:1). The pooled extracts were dried under N2, spotted on a reversed-phase plate (12), and developed in acetone/cholesteryl chloride (40:35, v/v). The radioactivity in the spots was determined by scraping the gel directly into vials to which Aquasol (New England Nuclear) was added for scintillation counting.

Other Methods—Protein was determined by the method of Lowry et al. (13) using bovine serum albumin as standard. Cholesteryl esters were eluted from Silica Gel G plates as described above and saponified by heating for 1 h at 65 °C with ethanolic KOH. Two volumes of hexane and one volume of water were added and the upper phase (sterol fraction) was dried for the determination of radioactivity.

RESULTS AND DISCUSSION

Time Course of Incorporation of ['H]Acetate into Cellular Cholesterol—Human foreskin fibroblasts synthesize radiocholesterol from labeled acetate. In agreement with previous reports (14), we found that the incorporation of radiolabeled acetate into cholesterol was dramatically enhanced if cells were preincubated in a medium containing lipoprotein-deficient serum. After 48 h of culture under these conditions, the synthesis of radiocholesterol was essentially linear over a period of several hours (Fig. 1).

Cholesterol oxidase was used to measure the time course of appearance of newly synthesized cholesterol at the plasma membrane. Incorporation into the plasma membrane initially lagged behind total synthesis (Fig. 1) but after about 2 h the rate of appearance of radiocholesterol at the cell surface became parallel to the rate of synthesis (steady state). These kinetics suggest that essentially all of the newly synthesized cholesterol migrates to the cell surface by a single pathway.

Our data can be analyzed in terms of a simple kinetic model to determine the rate of transfer of cholesterol from its site of synthesis to the plasma membrane. The model is described by

\[
['H]Acetate \rightarrow ['H]cholesterol \rightarrow \text{plasma membrane} \quad (1)
\]

where \(k_1\) and \(k_2\) are the first order rate constants describing cholesterol synthesis and the transfer of newly synthesized cholesterol to the plasma membrane, respectively. If the concentration of ['H]acetate remains constant during the experiment (so that the first step is a zero order process) and if the two processes described by \(k_1\) and \(k_2\) are irreversible, \(k_2\) can be estimated from the data shown in Fig. 1. It can be shown (see for example Ref. 15) that \(k_2\) is the reciprocal of the intercept with the abscissa indicated by the broken line in Fig. 1. Our data could be well described by the model in Equation 1 and yielded a value of 0.8 h\(^{-1}\) for \(k_2\) in the experiment shown in Fig. 1. This corresponds to a half-time of 0.86 h for the transfer of newly synthesized cholesterol to the plasma membrane in this experiment.

We used also a simpler method for determining \(k_2\) which did not depend on the lag and hence did not require determination of complete time courses like that shown in Fig. 1. Using the formalism given in Ref. 15, the fraction \(F\) of cholesterol in the plasma membrane at time \(t\) is given by:

\[
F = 1 + \frac{1}{k_2t} (e^{-kt} - 1) \quad (2)
\]

The per cent of cholesterol oxidized was plotted as a function of time and the value of \(k_2\) which best fit the data was determined for several experiments (Fig. 2). In four experiments, we found a half-time of (1.0 ± 0.1) h. This method had the advantage that variations between flasks in the incorporation of ['H]acetate into cholesterol did not affect the data since the parameter fit was the fraction susceptible to oxidation.
DeGrella and Simoni (7) studied the transfer of newly synthesized cholesterol to the plasma membrane in Chinese hamster ovary cells using a plasma membrane isolation method. They observed kinetics similar to those we report here; however, the half-time of transfer was only 10 min. This difference does not appear to be species-related since we observed similar time courses in both cell types (Fig. 2).

Rate of Movement of Cholesterol to the Plasma Membrane; Pulse-Chase Study—Cells in culture flasks were pulsed with [3H]acetate and chased by the addition of 10 mM unlabeled sodium acetate to the medium. At intervals thereafter, the cells were removed from the culture flasks and treated with cholesterol oxidase and the amounts of radioactivity associated with cholesterol and cholestenone were measured. The fraction of radiolabel susceptible to oxidation was found to increase with chase time at 37 °C but not at 4 °C, reflecting the transfer of newly synthesized cholesterol to the plasma membrane (Fig. 3B). The effectiveness of the chase was variable among experiments and was not always complete as seen by the continued incorporation of radiolabel into sterol after the initiation of the chase (Fig. 3A). This phenomenon, which also was observed by DeGrella and Simoni (7), may relate to the prolonged min-off of the numerous intermediates in the complex pathway of sterol biosynthesis. Nevertheless, we observed time courses similar to that shown in Fig. 3B in four experiments.

Careful kinetic analysis (Fig. 4) showed that the transfer in pulse-chase experiments was a strictly first order process with a final value of 95% of radiolabeled cholesterol in the plasma membrane. This value corresponds to the distribution of unlabeled cholesterol in these cells (5). The half-time of transfer was 2.0 ± 0.9 h (n = 4) at 37 °C. The difference between this half-time and that in the continuous labeling mode (Figs. 1 and 2) is presumably related to the variable effectiveness of the chase. It is noteworthy that there was no detectable lag in the transfer process following a 20-min pulse (Fig. 4).

The two methods used to estimate the rate of transfer of newly synthesized cholesterol to the plasma membrane...
yielded results which were in reasonable agreement given the assumptions involved in each of these experiments. Derivation of a half-time from the lag in the appearance of newly synthesized cholesterol at the plasma membrane (Fig. 1) depended on the assumption that the amount of [3H]acetate in the cells remained constant throughout the time course. In pulse-chase experiments (Figs. 3 and 4), it was assumed that the chase was totally effective. In both cases it was assumed that there was only one rate-limiting step: the time of transfer of radiolabeled cholesterol to the plasma membrane. The qualitative agreement of the two methods is reassuring although the shorter half-time, 1 h, is probably the more accurate. We conclude that the half-time of transfer is 1–2 h at 37 °C.

Effect of Cell Metabolism on Transfer of Cholesterol to the Plasma Membrane—The studies of transfer of newly synthesized cholesterol to the plasma membrane described above used confluent cells which had been preincubated for 48 h in lipoprotein-deficient serum to stimulate cholesterol synthesis (14). It also has been reported that cholesterol synthesis in rapidly dividing cells is enhanced relative to that in confluent cultures (16). To determine whether cellular metabolic state influenced cholesterol movement, we measured the rate of transfer of newly synthesized cholesterol to the plasma membrane in cells which had not been pre-exposed to lipoprotein-deficient serum, as well as in preconfluent monolayers.

Although incorporation of [3H]acetate into cholesterol was stimulated up to 300-fold by preincubating confluent monolayers of cells for 48 h in lipoprotein-deficient serum, the rate of transfer of newly synthesized cholesterol to the plasma membrane was not markedly different in starved cells and in cells maintained in the presence of serum. In a typical experiment (Fig. 2), the half-time of transfer in starved cells was 0.9 h compared to a value of 1.3 h for cells in serum. This difference, while significant, was not greater than the dispersion in half-time values measured in different experiments.

The rate of transfer of newly synthesized cholesterol to the plasma membrane in subconfluent cells was found to be similar to that measured in confluent monolayers (Table I). [3H]Acetate was added to the medium of cells which were either confluent (Table I, flasks 1–3) or less than 50% confluent (Table I, flasks 4–6). The fraction of newly-synthesized cholesterol susceptible to oxidation was determined after incubation of from 1 to 4 h at 37 °C. As can be seen from the table, the extent to which newly synthesized cholesterol was oxidized was similar for confluent and preconfluent cells at each time point. We conclude that the transfer of cholesterol to the cell surface is not strongly affected by the metabolic state of the cell but is inhibited at low temperature.

**Equilibrium Density Gradient Analysis of the Distribution of Newly Synthesized Cholesterol**—We sought to examine the subcellular distribution of cholesterol by sucrose density gradient fractionation. This analysis was made difficult by the fact that 95% of cellular cholesterol is in the plasma membrane (5) and that plasma membrane fragments are broadly distributed in such gradients, thus imposing considerable contamination on the cholesterol-poor internal membrane fractions. However, we found that this contamination can be eliminated by selectively converting plasma membrane cholesterol to cholestene by cholesterol oxidase treatment of the intact cells prior to homogenization. This system provides a sensitive approach to the study of the distribution of newly synthesized cholesterol in both intracellular and plasma membrane pools.

We traced the pathway of newly synthesized cholesterol through the cell by labeling cholesterol synthesized at two different times with two different radioisotopes. In one such experiment, cells were incubated with [14C]acetate for 2 h, the radiolabel was removed, and [3H]acetate was added for an additional 1-h incubation. The cells then were treated with cholesterol oxidase, homogenized, and spun on a sucrose density gradient. The amounts of each radiolabel in cholesterol and cholestene were determined throughout the gradient (Fig. 5). At the moment of cholesterol oxidase treatment, most of the cholesterol synthesized from the first added radiolabel (14C) had reached the plasma membrane, as evidenced by its extensive oxidation (panel A). However, only a small fraction of the cholesterol synthesized from the second added radiolabel (3H) had reached the plasma membrane, since most of it was unoxidized (panel B). Furthermore, we observe that the [3H]cholesterol profile in panel B showed a peak in fraction 4 not present in the [14C]cholesterol profile. This was a reproducible finding which may reflect the presence of intracellular membranes enriched in newly synthesized cholesterol.

**Is Exogenous Cholesterol Transferred from the Plasma Membrane to the Cell Interior?**—The plasma membrane can be labeled rapidly and selectively by introducing radiocholesterol into the medium (5). This exogenous probe initially was 95–97% susceptible to cholesterol oxidase, documenting its plasma membrane localization (5). The cholesterol oxidase method described above can potentially be used to examine the transfer of this cholesterol into the cell interior. However, since only about 5% of fibroblast cholesterol is intracellular, the disappearance of radiocholesterol from the plasma membrane into internal pools may not be reliably measured by the cholesterol oxidase method against a comparable background of unoxidizable plasma membrane radiolabel.

We approached this problem in two ways. In the first, we employed two different exogenous radiocholesterol probes to correct for incomplete oxidation of the plasma membrane. The strategy was to label the plasma membrane with exogenous [3H]cholesterol and allow several hours to elapse during which time the radiolabel potentially could equilibrate with internal pools. [14C]Cholesterol then was introduced in a like manner and the cells were immediately treated with cholesterol oxidase to determine the distribution of the two radiolabels between oxidizable and unoxidizable pools. The [14C] cholesterol provided an indicator of the plasma membrane cholesterol which was not oxidized, so that the excess of
unoxidized $^3$H over $^{14}$C would reflect transfer of $[^3]$Hcholesterol to internal pools. The results of a representative experiment are given in Table II. The first added radiocholesterol ( $^3$H) was 1–2% more susceptible to oxidation than the second added at all times. We do not take this tiny differential as significant, since it shows no time-dependent decrease as would be expected from a transfer process. Therefore, these data demonstrate that no significant transfer of $[^3]$Hcholesterol from the plasma membrane to the cell interior occurred during a 16-h incubation at 37°C.

The data in Table II notwithstanding, it could be that plasma membrane cholesterol does equilibrate with a subtraction of the internal cholesterol pool. To examine this possibility, the cell plasma membrane was labeled selectively by introducing $[^1]$Ccholesterol into the medium. The label was given the opportunity to equilibrate for several hours. Intracellular cholesterol was then labeled by the brief addition of $[^3]$Hacetate followed by a chase with unlabeled acetate.

**TABLE II**  
Transfer of exogenous cholesterol from plasma membrane to intracellular pools

| Incubation time (h) | Spot | Radioactivity (dpm) | Oxidation (%$^3$H/$^1$C) |
|---------------------|------|--------------------|--------------------------|
| 0                   | Cholesterol | 213 | 162 | 94 92 |
| 2                   | Cholest erone | 3480 | 1139 | 96 95 |
| 16                  | Cholesterol | 61 | 210 | 95 93 |
|                     | Cholestenone | 803 | 4409 |       |

**TABLE III**  
The distribution of exogenous $[^14]$Ccholesterol and newly synthesized $[^1]$Hcholesterol between plasma membrane and intracellular pools

Confluent fibroblasts were preincubated for 48 h at 37°C in medium containing 5% lipoprotein-deficient serum. The cells were labeled with $[^14]$Ccholesterol by the addition of the radioisotope in ethanol to the flask for 10 min as described (7). The medium was removed and the cells were incubated for 3 h to let the label equilibrate. Fresh medium containing 2.0 μCi of $[^1]$Hacetate was added. After a 45-min incubation at 37°C, the labeled medium was removed and replaced with medium containing 10 mM Na acetate. Following a 1-h incubation at 37°C, the cells were dissociated by trypsin treatment, treated with cholesterol oxidase, homogenized, and spun to equilibrium on a sucrose density gradient as described in the legend to Fig. 5. The radioactivity in cholesterol and cholestenone was determined for each fraction as described under "Experimental Procedures." In these double label experiments, external standards could not be used to correct for recovery after TLC; however, the recovery of the two radioisotopes in each spot is precisely determined.

 Plasma membrane cholesterol was oxidized by treating the intact cells with cholesterol oxidase to permit determination of the radiocholesterol from the two sources in cell surface and intracellular pools. Finally, the cells were homogenized and fractionated by sucrose gradient centrifugation. The use of two radioisotopes in these experiments made it possible to determine the ratio of their specific activities in the plasma membrane and cell interior without knowledge of the cholesterol mass in either pool. As shown in Table III, the ratio of exogenous to endogenous radioactivity (i.e., $^{14}$C/$[^3]$H) in choles-
tenone was much higher than in cholesterol in every fraction of the gradient. Thus, the two radiolabels did not equilibrate between the cell surface and any detectable intracellular pool. These data therefore are inconsistent with a cholesterol transfer process involving one-for-one exchange of plasma membrane cholesterol with a substantial pool of intracellular cholesterol.

Esterification of Exogenously Incorporated 
$[^{14}C]$Cholesterol—The mingling of plasma membrane cholesterol with intracellular pools also can be examined by measuring its availability for esterification. The intracellular esterification of cholesterol is mediated by acyl-CoA:cholesterol acyltransferase, an enzyme which is associated with the endoplasmic reticulum (17). Poznansky and Czekanski (6) reported that in human skin fibroblasts, exogenously introduced cholesterol was not esterified during a 24-h incubation. In contrast, Slotte and Lundberg (18) showed esterification within 4 h of cholesterol introduced into smooth muscle cells from cholesterol-enriched sonicated liposomes.

We used this approach to compare the availability of exogenously introduced $[^{14}C]$cholesterol and newly-synthesized $[^{3}H]$cholesterol for intracellular esterification. Cells in culture flasks were labeled with $[^{14}C]$cholesterol by adding the radiolabel to the medium and then incubated with $[^{3}H]$acetate for 1 h at 37 °C. The label was removed and chased for 1 h at 37 °C with 10 mM Na acetate; the cells then were harvested and the radioactivity in free and esterified cholesterol was measured. The biosynthetic $[^{3}H]$cholesterol was preferentially incorporated into the cholesteryl ester pool: 28% of cellular $[^{3}H]$cholesterol was esterified compared to only 0.5% of the $[^{14}C]$cholesterol (Table IV, experiment 1). Furthermore, incubation of the exogenously labeled cells for periods up to 18 h (Table IV, experiment 2A) did not result in significant esterification of the exogenous radiolabel. These data support the hypothesis that plasma membrane cholesterol does not equilibrate with newly synthesized cholesterol within the cell and that cholesterol movement from plasma membrane to intracellular pool occurs extremely slowly, if at all.

Brown and Goldstein (10) showed that the addition to the medium of 2–15 μg/ml cholesterol in ethanol suppressed 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured fibroblasts during a 24-h incubation, suggesting that exogenous cholesterol entered the cell. This finding must be reconciled with our observation that exogenously added radiolabeled cholesterol is transferred slowly, if at all, from the plasma membrane to the cell interior. The explanation may lie in the relatively high concentration of cholesterol added in the study cited (10). Because cholesterol forms aggregates in aqueous solution above about 10−8 M (19), it is possible that in these experiments the sterol entered the cell by endocytosis and not by a molecular transfer mechanism. We tested this hypothesis by examining the esterification of exogenous $[^{14}C]$cholesterol in the presence of added cholesterol carrier. Unlabeled cholesterol was added to the medium in the culture flask together with $[^{14}C]$cholesterol and $[^{3}H]$acetate and the cells were incubated for 18 h at 37 °C. The addition of 10 μg/ml unlabeled cholesterol to the medium led to a 6-fold increase in the incorporation of the exogenous label into cholesteryl esters (Table IV, experiment 2A). A concomitant reduction of approximately 40% in the esterification of the synthesized cholesterol was observed as expected (10). These data suggest that above a critical threshold concentration, cholesterol can be internalized by the cells even though trace amounts of radiolabeled cholesterol are not transferred from the plasma membrane to intracellular pools.

### Table IV

| Experiment | Flask | Cholesterol added | Total protein | Fraction | Radioactivity | Esterification |
|------------|------|------------------|--------------|----------|---------------|----------------|
|            |      |                  |              |          | $[^{1}H]$ | $[^{1}H]$-C | $[^{1}H]$-C |
|            |      |                  |              |          | dpm          |                |
|            |      |                  |              |          | %            |                |
| 1          | 1    | 0                | N.D.* Cholesterol | 677 | 2,708 | 28 | 0.5 |
| 2          | 2    | 0                | Cholesterol | 135,557 | 273,497 | 0.9 | 0.3 |
| 3          | 3    | 0                | Ester | 1,178 | 791 |
| 4          | 4    | 0                | Cholesterol | 163,309 | 226,630 | 0.8 | 0.9 |
| 5          | 5    | 0                | Ester | 1,246 | 2,123 |
| 6          | 6    | 0                | Cholesterol | 165,134 | 227,025 | 0.6 | 1.9 |
| 7          | 7    | 0                | Ester | 1,100 | 4,436 |

* Not determined.

### CONCLUDING COMMENTS

The mechanism by which the striking nonuniformity of cholesterol in mammalian cell membranes is maintained can now be understood in terms of a simple working hypothesis. Plasma membrane cholesterol is in equilibrium with plasma lipoproteins; indeed, in erythrocytes it appears that membrane cholesterol levels are determined primarily by plasma-esterified cholesterol (20, 21). Despite the internal synthesis of cholesterol in tissue cells, it is conceivable that their plasma membrane cholesterol is similarly a function of rapid equilibration with plasma lipoproteins. It has been reported that cholesterol transfer between red cells and plasma lipoproteins involves diffusion of the sterol through the aqueous phase (22, 23). However, the movement of radiolabeled cholesterol between red cells is negligibly slow compared to its transfer to lipoproteins (24), suggesting that it cannot simply be released into the aqueous medium. Furthermore, Wulffenberg and Silber (25) have shown that the cholesterol in various subcellular fractions of LM cells is not in diffusional equilibrium. The data presented here suggest that cholesterol does not diffuse between plasma membranes and internal organelles, in that exogenously introduced radiolabel does not reach intracellular pools during the time its takes newly synthesized cholesterol to reach the plasma membrane. Rather, cholesterol appears to be transported unidirectionally from its site of synthesis to the plasma membrane with a half-time of 1–2 h at 37 °C.

The mechanism by which newly synthesized cholesterol is transferred to the plasma membrane remains to be elucidated.
Cytoplasmic proteins such as those which promote intermembrane transfer of certain intermediates in cholesterol and steroid biosynthesis (26, 27) could be involved. However, we have been unable to detect newly synthesized cholesterol in the soluble protein fraction of cell homogenates. We feel that it is more likely that newly synthesized cholesterol is transported to the plasma membrane by specific membrane vesicles as has recently been suggested for phospholipids (3). If this is the case, our demonstration of first order kinetics and no detectable lag in the appearance of newly synthesized cholesterol at the plasma membrane may signify that the insertion of this cholesterol into transport vesicles is not rate-limiting in the overall process; rather, delivery of the cholesterol to the cell surface by such vesicles could be the slow step. That is, the first order time course suggests that the bulk of intracellular cholesterol constitutes a single pool in rapid equilibrium compared with the unidirectional transfer to the cell surface.

Our experiments using serial additions of \(^{14}C\)acetate and \(^{3}H\)acetate to the medium of cells in culture showed that newly synthesized cholesterol was chased from a high density fraction into a lower density fraction (Fig. 5). The high density membranes (-1.22 g/ml) could derive from endoplasmic reticulum since this organelle is the site of cholesterol synthesis (28) but also could be a transport vesicle. It is noteworthy that the low density fraction to which newly synthesized cholesterol moved prior to becoming available for oxidation generally co-migrated with one of the major plasma membrane peaks (Fig. 5). While this juxtaposition could be fortuitous, it also could indicate the association of vesicles containing newly synthesized cholesterol with the plasma membrane prior to actual delivery, perhaps by fusion. Accessibility to cholesterol oxidase in intact cells would signify the incorporation of this new sterol into the plasma membrane.

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