Macrophage cholesterol exchanges with cholesterol of serum lipoproteins (1). Kinetically, the exchangeable cholesterol of the macrophage fits a model of two compartments linked in series to a large reservoir. Although kinetically distinguishable compartments do not necessarily have a morphological counterpart (2), most of the macrophage cholesterol can be accounted for in plasma membrane and lysosomes (1). Thus it is possible that the rapidly exchanging compartment is the plasma membrane, and the more slowly exchanging compartment is the intracellular membrane, mostly lysosomal. This hypothesis can be tested by manipulating the putative compartments in known ways. In this article we examine the effects of enzymatic modification of the plasma membrane, as well as increasing the pool of intracellular lysosomal membrane on the content and compartmentalization of macrophage cholesterol. In addition, a compartmental analysis of other cultured cells with different proportions of cholesterol-rich cytomembranes will be presented.

Materials and Methods

Normal mouse peritoneal macrophages were cultivated in newborn calf serum (NBCS)2 in medium 199 (TC 199) as described previously (1, 3). Cells were labeled by exchange with serum lipoproteins equilibrated with cholesterol-7α-3H (1). Procedures for gas-liquid chromatographic analysis of cholesterol, thin-layer chromatography, and enzymatic and analytic procedures have been described elsewhere (1).

Cell Cultivation. —

Thioglycollate-stimulated mouse peritoneal macrophages (4) were produced in vivo by injecting 25 g NCS mice intraperitoneally with 0.6 ml of a thioglycollate broth (2.98 g/100 ml, [...]}
Difco Laboratories, Detroit, Mich.) which had been autoclaved, then aged for 2 wk at 4°C. 4 days later peritoneal cells were collected in heparinized phosphate-buffered saline (PBS). About 15-20 × 10^6 cells were obtained from each mouse. After centrifuging at 800 rpm, the cells were resuspended in 10% NBCS in TC 199 to a final concentration of 2 × 10^6 per ml, and 2.0 ml placed on 30-mm diameter plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.). After 1 hr at 37°C, nonadherent cells were removed, and the macrophages washed two times with TC 199. Fresh serum medium was added, and the cells were replaced in the CO₂ incubator. Thioglycollate macrophages were fractionated by differential centrifugation as described previously for unstimulated macrophages (1).

Calmette-Guérin bacillus (BCG)-induced rabbit alveolar macrophages were kindly supplied by Dr. Ralph Nachman, and were obtained by the method of Cohn and Wiener (5), 3-4 wk after intravenous injection of lyophilized BCG. The intact cells were washed twice with PBS and resuspended at a concentration of 1 × 10^6 cells/ml in minimal essential medium (MEM, Microbiological Associates, Inc., Bethesda, Md.) containing 10% fetal calf serum (FCS). Cells were incubated in sterile polypropylene tubes, after gassing with 5% CO₂-air, on a shaking platform at 37°C. Cell viability, as determined by Trypan blue exclusion, remained at greater than 95% for more than 16 hr.

L-929 strain mouse fibroblasts were kindly provided by Dr. S. Silverstein. The cells were placed on 30-mm Falcon plastic dishes at a concentration of 5 × 10^5/ml in MEM supplemented with 10% FCS. They were then grown overnight to confluency.

Mouse melanoma cells, originally derived from a nonpigmented clone (6), were kindly provided by Dr. S. Gordon. They were placed on 30-mm Falcon dishes in 10% FCS in MEM after trypsinization and grown overnight to confluency at 37°C in a CO₂ incubator.

Microscopy.—Cells on cover slips were fixed in glutaraldehyde and examined under oil immersion phase illumination as described previously (3). Electron micrographs were kindly taken by Doctors James G. Hirsch and Martha E. Fedorko. Cells in flasks or Petri dishes were fixed and embedded in Epon as described by Hirsch and Fedorko (7). Thin sections, stained with uranyl acetate and lead citrate (8), were examined in a Siemens Elmiskop I (Siemens America, Inc., New York) at 80 kv using a 50 µ objective aperture.

Analytic Procedures.—Protein was assayed by the method of Lowry et al. (9) using egg white lysozyme as standard. For phospholipid phosphorus, phospholipids were extracted into chloroform-methanol (10). The lipid phase was taken to dryness and the phospholipids were ashed with Mg(NO₃)₂ (11). Phosphorus was determined by comparison with dipalmitoyl lecithin-phosphorus and inorganic phosphate standards, after addition of ascorbic acid and molybdate color reagent (12).

Experiments Using Polystyrene Latex.—Polystyrene latex particles 1.099 µ in diameter (Dow Chemical Co., Midland, Mich.) were washed in 50 volumes TC 199, resuspended in 20% NBCS at a final concentration of 500 µg/ml, and added to macrophage cultures. Phagolysosomes were isolated from macrophages which had ingested polystyrene latex particles using the method of Wetzel and Korn (13) as modified by Werb and Cohn.¹

Other Materials.—Polystyrene latex particles 1.099 µ in diameter, Dow Chemical Co.; lyophilized trypsin, Worthington Biochemical Corp., Freehold, N.J.; dextran 2000 and dextran sulfate 2000, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; puromycin dihydrochloride and yeast invertase, Nutritional Biochemical Corporation, Cleveland, Ohio; sodium α-naphthyl acetate and lead citrate (8), were examined in a Siemens Elmiskop I (Siemens America, Inc., New York) at 80 kv using a 50 µ objective aperture.

All other chemicals were commercially available analytic reagent grade. Solutions added to cell cultures were prepared immediately before use and sterilized by passage through 0.45 µ Millipore filters (Millipore Corp., Bedford, Mass.).

Mathematical Methods.—Procedures for measurement and calculation of kinetic parameters and pool sizes have been described previously (1, 14).
RESULTS

Macrophages are highly active endocytic cells which may interiorize large amounts of plasma membranes are pinocytic and phagocytic vacuoles (3, 5, 15-18). After fusion with preexisting lysosomes, the endocytic vesicles are converted into secondary lysosomes (15-17, 19). To maintain the size and content of these structures, nondigestible materials of either soluble or particulate nature may be employed. In this study we use nondegradable disaccharides and polysaccharides as well as polystyrene beads to interiorize plasma membrane and enrich the intracytoplasmic pool of secondary lysosomes (20, 21).

For the purpose of modifying the cell surface, brief digestion with trypsin was employed. This procedure has the advantage of not detaching the cells from the glass surface and not influencing the composition of intracellular compartments.

Effect of Trypsin.—The exposure of macrophages to 50–1000 µg trypsin/ml in TC 199 leads to reversible morphological changes as documented in Fig. 1 a and b. When the cells are replaced in 20% NBCS, recovery begins within 15 min and by 1 hr mitochondria return to the cell periphery. The effect of trypsin on the kinetics of cholesterol exchange was studied by 3H-uptake experiments after trypsin treatment, or by washout from macrophages prelabeled with cholesterol-3H and then trypsinized.

Macrophages, prelabeled for 4 hr in 20% NBCS containing 1 µCi/ml of cholesterol-7-3H, were exposed to either 500 µg trypsin/ml medium or TC 199 alone for 1 hr. No phospholipid phosphorus, free cholesterol, or tritium label was lost during the trypsin treatment, and cell viability was 100% (Fig. 2). The washout of label into 20% NBCS is shown in Fig. 2 a. A decrease of at least 50% in the rate of exchange occurred in the trypsin-treated cells. This reduced rate was evident for the first 7 3/4 hr (7.3 ± 0.2 hr for six experiments) and was followed by an abrupt resumption of exchange.

The effect of trypsin on cholesterol exchange was in both directions. Uptake of label was reduced to 40–60% of control values as measured by a continuous exposure to radioactive medium for 6 hr. This decrease was also evident in 1-hr pulses of label up to the end of the 7th hr; then during the 8th hr the incorporation in a 1 hr pulse returned to control values.

The “recovery” of control rates of exchange was sensitive to inhibitors of protein synthesis. Cycloheximide (2 µg/ml) or puromycin (2–10 µg/ml) inhibited the recovery phase (Fig. 2 b). The washout continued at its slow rate long after recovery was complete in the cells without inhibitor. Control cells also showed an effect of puromycin treatment after prolonged exposure (Fig. 2 c).

Experiments were carried out to test whether the trypsin-sensitive site required a serum constituent for reconstitution. Labeled macrophages were
trypsinized and incubated for 4 hr in the presence of 1–35% NBCS. The medium was then changed to 20% NBCS and the cells were observed for the next 6 hr. In all cases recovery occurred at about 7½ hr after trypsinization with no change in rate indicative of the influence of a serum factor.

Effect of Intralysosomal Storage of Pinocytized Dextran and Dextran Sulfate.—

Cultivation of macrophages in medium containing nondegradable molecules results in the accumulation of many phase-lucent granules (Fig. 3). Cells maintained in vitro for 72 hr in 20% NBCS supplemented with 10 μg dextran sulfate/ml (mol wt = 2 × 10^6 daltons), 10 mg dextran/ml (mol wt = 2 × 10^6 daltons) have many more phase-lucent vacuoles or secondary lysosomes found in the perinuclear zone than do controls. Cells cultivated in medium supplemented with dextran sulfate (10 μg/ml) for at least 48 hr exhibited a slower rate of uptake of cholesterol-3H than controls. Dextran-laden cells

![Image](image-url)
FIG. 2. The effect of trypsin on kinetics of cholesterol exchange. (a) Washout of cholesterol-$^3$H into 20% NBCS from prelabeled macrophages treated for 1 hr with 500 μg/ml trypsin. (b) Effect of puromycin on washout of label from trypsin-treated macrophages. (c) Effect of puromycin on washout of label from control macrophages.

were also studied to control for possible binding of lipoproteins to dextran sulfate (22).

Uptake of cholesterol-$^3$H by macrophages grown for 72 hr in dextran or dextran sulfate was similar. On a protein basis the uptake was slower than for
controls, but reached control values after 6 hr of incubation. Kinetically, this indicated that $K_s$, the time zero contribution of the slower exponential, was larger, and hence the slowly exchanging compartment was larger than in control cells.

Washout of label from the dextran and dextran sulfate-laden cells, pre-

![Fig. 3. Morphology of macrophages cultivated in the presence of nondegradable solutes for 72 hr. Phase contrast $\times$ 1250. (a) A cell which has been cultivated in 20% NBCS with 10 $\mu$g/ml dextran sulfate. Phase-lucent vacuoles fill the cytoplasm. Only the Golgi region, in the hof of the nucleus, is free of vacuoles. Highly refractile lipid droplets are present outside the granule region. (b) A cell which has been cultivated in 20% NBCS supplemented with 10 mg/ml dextran. The cytoplasm is filled with clear vacuoles. Some phase-dense granules are present as well. The rodlike mitochondria extend into the pseudopods. (c) This cell was cultivated in 20% NBCS containing 10 mg/ml sucrose. Many phase-lucent lysosomes are present. There are several layers of vacuoles although only one layer can be seen in a micrograph. (d) This control cell cultivated in 20% NBCS contains small phase-dense granules and a few phase-lucent pinocytic vesicles.

labeled for 4 hr, into either 10 or 20% FCS proceeded more slowly than from control cultures (Fig. 4). The change in over-all rate was not due to a change in the time constants of the two exponential phases, but to an increase in amount of label contributed by the slower phase. The cells cultivated in dextran or dextran sulfate medium had increased cellular cholesterol levels, and increased cholesterol to protein ratios (Table I).

The membrane area of the cells was measured directly by planimetry from phase micrographs of well spread cells enlarged 2000 times. The plasma membrane area was estimated as
twice the area of the surface, and the granule area from the number and surface area of the granules. At least 100 cells of each type were measured in two independent experiments. Control cells contained about 50 granules of 0.2-0.5 μ in diameter, dextran sulfate-laden cells contained about 200–250 vacuoles of about 1 μ diameter, dextran-laden cells contained about 150 1.2-μ granules, and sucrose-laden cells contained about 250 1.5-μ granules. The measured plasma membrane area was about 2–3 \times 10^2 \mu^2 in all four cases. Errors in both figures were

![Graph showing washout of cholesterol-3H label from dextran or dextran sulfate-laden macrophages. Macrophages were cultivated for 72 hr in 20% NBCS only, or supplemented with 10 mg/ml dextran or 10 μg/ml dextran sulfate, labeled for 4 hr in 10% FCS containing 1 μCi/ml cholesterol-3H, and then placed into isotope-free 10% FCS.](image)

in the direction of underestimates. Cells filled with storage vacuoles were often more rounded than unvacuolated controls and the surface contained many invaginations, projections, and ruffles. Only the layer of granules seen in one optical plane was measured. By focusing through cells, it was obvious that most cells had four or more layers of granules. Another error in the granule area calculation occurred because granules less than 0.1 μ in diameter could not be resolved, and were ignored in the calculation. This underestimate was particularly striking in the control cells. However, despite these limitations, the proportion of the membrane area as granule area served as an estimate of the increment in lysosomal membrane.
### Table I

**Effect of Lysosomal Storage of Nondigestible Solutes on Cholesterol Content, Membrane Area, and Compartment Sizes**

| Substance stored in lysosomes* | Cholesterol | Membrane area | Plasma membrane | Compartmental analysis† | Exchangeable cholesterol compartments |
|------------------------------|-------------|---------------|-----------------|-------------------------|---------------------------------------|
|                              | µg/flask‡ | % control | µg/mg protein | µ^a x 10^3 | % total | % | hr | % | hr | % total | µg C | % total | µg C |
| Dextran                      | 2.44 (6)  | 133 (a)    | 16.2           | 2.2 (50)‡ | 82 | 20 | 3.0 | 80 | 15.0 | 44.2 | 1.08 | 55.8 | 1.36 |
| Dextran sulfate              | 1.79 (5)  | 123 (a)    | 15.0           | 3.6 (50)  | 77 | 25 | 2.6 | 75 | 14.6 | 46.3 | 0.83 | 53.7 | 0.96 |
| Sucrose                      | 4.96 (3)  | 185 (b)    | 24.9           | 2.3 (50)  | 68 | 18 | 4.5 | 82 | 28.5 | 47.7 | 2.37 | 52.3 | 2.59 |
|                             | 3.31 (3)  | 166 (c)    | 25.7           |           |    | 40 | 1.3 | 60 | 14.5 | 52.5 | 1.64 | 47.5 | 1.49 |
| Control                      | 1.84 (6)  | 100 (a)    | 11.9           | 2.5 (50)  | 97 | 55 | 2.3 | 45 | 18.0 | 66.2 | 1.22 | 33.8 | 0.62 |
|                              | 2.65 (4)  | 100 (b)    | 13.1           |           |    | 54.5 | 4.1 | 45.5 | 20.5 | 71.8 | 1.90 | 28.2 | 0.75 |
|                              | 1.87 (3)  | 100 (c)    | 12.9           |           |    | 60 | 1.6 | 40 | 15.0 | 68.4 | 1.28 | 31.6 | 0.59 |

* Macrophages were exposed to solutes as follows: dextran, 10 mg/ml in 20% NBCS, 72 hr; dextran sulfate, 10 µg/ml in 20% NBCS, 72 hr; sucrose, 10 mg/ml in 20% NBCS, 72 hr; control, 20% NBCS, 72 hr.

† Compartmental analysis of the die-away kinetics is performed by the method described previously (1). The relative sizes of the fast and slow pools are calculated in per cent total, and in µg cholesterol/flask culture.

‡ Numbers in parentheses indicate number of samples.

§ Letters indicate matched experiments.
The contribution of granule membrane area to the total membrane area increased from 3% in the controls to 20% in the dextran and dextran sulfate-filled cells (Table I). The slowly exchanging compartment increased from 30% of the cholesterol in the controls to 50% of the cholesterol in the laden cells.

The increase in the sizes of this compartment therefore accounted for most of the increment in cholesterol.

**Effect of Intralysosomal Storage of Sucrose.**—Macrophages grown in the presence of 10 mg/ml sucrose for 72 hr were filled with phase-lucent secondary lysosomes (20) (Fig. 3). There appeared to be more vacuoles in the sucrose-laden cells than in the dextran and dextran sulfate cells. The total incorporation of cholesterol-3H into the sucrose-laden cells was the same as into the
controls. Kinetics of label washout during the subsequent 20 hr in 20% NBCS are shown in Fig. 5. The sucrose-laden macrophages exchanged their cholesterol more slowly than control cells. The decrease in overall rate was due to an increase in the contribution of the slowly decaying exponential phase and the time constants were not significantly changed (Table I).

Macrophages with sucrose-filled storage granules contained twice as much cholesterol as control cells (Table I). The measurable granule area represented 40% of the total area and the slowly exchangeable cholesterol pool increased to 50% of the total. This increase accounted for most of the increase in cholesterol. Phospholipid phosphorus and acid phosphatase were also increased in sucrose-laden cells. Flask cultures contained 3.02 μg cholesterol, 26.3 nmoles phospholipid, and 43.2 nmoles α-naphthol released/min acid phosphatase activity in the sucrose-laden cells, compared to 1.87 μg cholesterol, 11.8 nmoles phospholipid, and acid phosphatase activity of 22.4 nmoles α-naphthol/min for control cultures. This also suggested that an increase in secondary lysosomes had taken place.

Alteration of Cellular Membrane Distribution by Phagocytosis of Latex Particles.—12 hr after macrophages had taken up 1.1 μ latex particles (5 $\times$ 10$^8$/

![Image](image.png)

**Fig. 6.** Phase-contrast appearance of a macrophage which has ingested 1.1 μ latex particles. 24 hr after ingestion the refractile particles are arranged in the perinuclear area. The peripheral cytoplasm is well spread and contains rodlike mitochondria. × 1500.
ml, 1 hr) (Fig. 6), they were labeled with cholesterol-3H in 20% NBCS for 5 hr. The kinetics of washout of label was followed (Fig. 7). The over-all loss of label from latex-filled cells was slower than control, and the relative contribution of the fast and slow exponential phases had been altered. The calculated size of the slowly exchanging compartment had increased (Table II), and the cholesterol content was accounted for by the increase in the slow pool size. Uptake of label proceeded at rates comparable to controls, resulting in

![Graph](image)

**Fig. 7.** Washout of cholesterol-3H label from latex-laden macrophages. Cells were exposed to 1.1 μ latex particles for 1 hr, washed, then incubated in 20% NBCS for 12 hr. The cells were exchange labeled with cholesterol-3H for 5 hr, then loss of label in 20% NBCS followed.

the same specific activity of cholesterol, although the total incorporation per culture was greater in the cultures which had ingested latex.

Kinetics of cholesterol-3H washout performed immediately after phagocytosis indicated that the rapidly exchanging compartment was decreased in size and rate for the first 5 hr. Rigorous interpretation of these kinetics was difficult because a 50% increase in cell cholesterol occurred between 5 and 10 hr after phagocytosis. 1

*Contribution of Membrane Flow to Labeling of the Intracellular Cholesterol Compartment.*—Isolation of phagolysosomes after exchange labeling in the presence and absence of fluoride was used to determine the contribution of the pinocytic flow of plasma membrane to the labeling of the intracellular or
slowly exchanging cholesterol compartment. Macrophages were allowed to phagocytize 1.1 μ latex particles for 1 hr, washed to remove extracellular beads, then incubated in 20% NBCS for 12 hr. The cells were exchange labeled for 5 hr in 50% NBCS containing 1 μCi/ml cholesterol-3H. Both pinocytosis and phagocytosis were inhibited in one set of cultures by addition of 5 X 10⁻³ M sodium fluoride (23). The phagolysosomes isolated from cells incubated in the inhibitor contained the same specific activity of cholesterol as those from control cells. Thus membrane flow during pinocytosis is probably a minor component in the labeling of the slow or intracellular compartment.

**BCG-Induced Rabbit Alveolar Macrophages.**—The alveolar macrophage has large numbers of morphologically heterogeneous secondary lysosomes (Fig. 8 a) which are the end products of phagocytic and pinocytic events. The cells could be maintained in 10% FCS in MEM for more than 20 hr with greater than 95% viability, and no change in cell protein or cholesterol content. Alveolar macrophages contain about twice as much cholesterol on the basis of protein, and nearly ten times as much cholesterol per cell as compared to peritoneal macrophages.

Kinetics of label uptake in 10% FCS proceeded linearly for about 4 hr and then became asymptotic (Fig. 9 a). On a cholesterol basis the isotopic equilibrium specific activity of the cells was virtually identical to that of the medium (10⁶ dpm/μg cholesterol). After a 14½ hr incubation in radioactive medium, the cells were resuspended in isotope-free medium. The washout of label, plotted semilogarithmically, is shown in Fig. 9 b. The rapid exponential phase had a shorter duration than in peritoneal macrophages. The kinetic parameters gave a calculated size of the slowly exchanging compartment of

### TABLE II

| Experiment* | Cholesterol content| Graphical analysis | Rapidly exchanging pool | Slowly exchanging pool |
|-------------|-------------------|-------------------|-------------------------|------------------------|
|             | μg/flask | μg/mg protein | % | % | % | μg | % | μg |
| Latex       | 1.85 (6) | 1.81 | 25 | 1.4 | 75 | 15.5 | 39.5 | 0.75 | 60.5 | 1.10 |
| Control     | 1.20 (4) | 1.25 | 50 | 1.5 | 50 | 13.0 | 60.9 | 0.73 | 39.1 | 0.47 |

* Macrophage flask cultures were exposed to 5 X 10⁶ latex particles/ml in 20% NBCS for 1 hr, washed then cultivated for 8 hr. Cells were then labeled with cholesterol-3H, 1 μCi/ml, in 20% NBCS for 4 hr, then the washout of label was followed. Control cultures were treated similarly, but the exposure to latex was omitted.

† Number in parentheses indicates number of determinations.
54%, which was almost twice that for peritoneal macrophages. Similar results were obtained from analysis of uptake curves and in the presence of normal rabbit serum. Since the alveolar macrophages had twice as much cholesterol on a protein basis as peritoneal macrophages (Table III), most of this increase was contributed by the slow pool, although a significant increase in the cholesterol of the fast pool also occurred.

*Thioglycollate-Stimulated Mouse Peritoneal Macrophages.*—Thioglycollate cells exhibit numerous dense cytoplasmic granules and large vacuoles (Fig. 8 b). These cells spread out more readily on plastic dishes, and contained three to four times the cholesterol per cell than macrophages stimulated in vitro for 48 hr in 20% NBCS (Table III). The increased cholesterol was in the form of free cholesterol, and no cholesterol ester was detectable. The uptake of label was linear for 5 hr, then slowed to a new linear rate for the next 20 hr, and finally approached the equilibrium value (Fig. 9 c). The kinetics of washout after a 12 hr labeling period are shown in Fig. 9 d. The die-away curve decreased much more slowly than for unstimulated macrophages. The slowly exchanging compartment contributed half of the cellular cholesterol in these cells (Table III) as calculated from both uptake and washout data. If the absolute pool sizes relative to protein are calculated, it can be seen that the increase in cholesterol is in the slow pool, while the fast pool contributes about the same amount as normal macrophages. Since the morphologically assessable number of secondary lysosomes has been increased, it is likely that the cholesterol increase is due to the increase in cholesterol-containing lysosomal membrane.

To elucidate further the nature of the increase in cholesterol, cell fractionation studies were performed. The large granule fraction, which contained 70% of the acid phosphatase, was enriched fourfold in cholesterol, and contained 50% of the label after a 16 hr labeling period, although the specific activities...
Fig. 9. Cholesterol exchange kinetics. (a, b) Kinetics of cholesterol exchange in the rabbit alveolar macrophage. (a) Uptake of cholesterol-^3H from labeled lipoproteins in 10% FCS. (b) Washout of cholesterol-^3H from alveolar macrophages prelabeled for 141/2 hr. 5 X 10^6 cells contain about 80 μg protein. The dashed line 1 shows the fast exponential phase, and the dashed line 2, the slow exponential phase. (c, d) Kinetics of cholesterol exchange in mouse thioglycollate-stimulated macrophages. (c) Uptake of cholesterol-^3H from labeled lipoproteins in 10% FCS from cells prelabeled for 12 hr. The dashed line marked 1 shows the rapid exponential component, and the line 2 shows the slow exponential component. (e) Uptake of label into L-cells from cholesterol-^3H-labeled 10% FCS medium. (f) Uptake of cholesterol-^3H label into melanoma cells grown in 10% FCS.
in all the fractions were similar. When cells were labeled for 6 hr followed by a 10 hr washout period, the specific activity of cholesterol increased to 140% of the homogenate value, while the crude nuclear and microsomal fractions, which contained much of the plasma membrane, decreased to 70% of the medium specific activity, in keeping with the two-pool model proposed previously (1).

**L-929 Strain Mouse Fibroblasts.**—L-cells differ from macrophages in having very few endocytic vesicles and secondary lysosomes, and contain larger amounts of endoplasmic reticulum (Fig. 8 c). Kinetics of cholesterol-\(^{3}H\) uptake proceeded linearly for 8 hr then leveled off approaching an asymptote (Fig.

**TABLE III**

| Cell type                        | Cholesterol/protein* | Exchangeable cholesterol compartments |
|----------------------------------|----------------------|----------------------------------------|
|                                  | µg/µg    %     %    | Fast pool  Slow pool Slow/fast         |
| Normal mouse peritoneal macrophage | 12     69      31     | 0.45                                   |
| Thioglycollate-stimulated mouse macrophage | 18     51      49     | 0.96                                   |
| Rabbit alveolar macrophage       | 25     46      54     | 1.17                                   |
| L-929 cell                       | 6.5    81-87‡  13-19‡ | 0.2                                    |
| Melanoma cell                    | (1.5)§  >95‡   <5§     | <0.05                                  |

* Normal macrophages, 0.2-0.5 µg free cholesterol (FC)/10^6 cells; thioglycollate macrophages, 0.5-2.0 µg FC/10^6 cells; alveolar macrophages, 3.1 µg FC/10^6 cells; L-929 cells, 0.2 µg FC/10^6 cells; melanoma cells, ~0.08 µg FC/10^6 cells.

† Long-term isotopic washout experiments could not be performed for these growing and dividing cells. Pools were estimated from uptake and partial washout curves.

§ Detectable, but too small to measure accurately.

∥ A slowly exchanging compartment could not be detected.

Washout experiments were difficult to perform because these cells continued to grow and divide during the experiment. However, the washouts of short duration showed the presence of a slow component representing a \(K_a\) (time zero intercept of the slow exponential) of less than 30%. Calculation of the relative pool sizes from uptake and washout data indicates that about 10-20% of the cell cholesterol was present in the slowly exchanging cholesterol compartment (Table III). Since the cholesterol content of L-cells is less than that of peritoneal macrophages, the difference in cell cholesterol appears to be due to a smaller contribution of the slow intracellular compartment.

**Mouse Melanoma Cells.**—Melanoma cells have many polyribosomes, lipid droplets, and mitochondria, but little smooth-surfaced membrane identifiable as the cholesterol-rich type (Fig. 8 d). Uptake of label on a cell protein basis was much less than for macrophages exposed at the same time and was re-
CHOLESTEROL COMPARTMENTS

lated to their low cholesterol content (Table III). Rate of uptake of label was linear for 7 hr (Fig. 9f). Washout experiments could only be carried out over short periods because overgrowth of the cultures occurred. Only a single exponential component was evident and extrapolation of the uptake curve also indicated only a single exponential. Thus only one distinguishable cholesterol compartment was present although a slow exponential phase of less than 5% would not be detected.

DISCUSSION

Morphological Identification of the Rapidly Exchanging Cholesterol Compartment in Macrophages.—Trypsin acts on the plasma membrane of cells removing a number of peptides (24–26), while maintaining the integrity of the cell surface. The macrophage remained firmly attached to its substrate, retained its ability to phagocytize, and exhibited rapidly reversible morphological changes. However, kinetics of cholesterol exchange were markedly altered. The rate characteristic of the rapidly exchanging compartment was reduced 40–90%, although no cholesterol was released by trypsinization. This suggests that a membrane-associated lipoprotein is not removed by trypsin. The trypsin-labile site may be involved in positioning lipoproteins at the cell surface, so that efficient exchange can take place. It requires 7 hr for the “receptor” to be replaced, a time period which may represent the synthesis of new receptors and their insertion into the membrane. In any case the regeneration of receptors is an active process which requires ongoing protein synthesis. In nontrypsinized cells, the inhibition of protein synthesis reduced receptor activity after 5 hr. During protein synthesis inhibition intracellular pools of receptor proteins may be exhausted and not replaced, while turnover occurs at normal rates. The action of trypsin at the level of the cell surface is consistent with the plasma membrane localization of the rapidly exchanging cholesterol compartment.

Morphological Identification of the Slowly Exchanging Cholesterol Compartment in Macrophages.—Factors affecting the relative distribution of cholesterol-rich cytomembranes in the macrophage altered the relative sizes of the rapidly and slowly exchanging cholesterol compartments. Extensive pinocytosis and phagocytosis of substances resistant to macrophage lysosomal hydrolases resulted in the increased number and size of secondary lysosomes. Increased levels of lysosomal hydrolases also were evident as a result of pinocytosis, but not phagocytosis (27, 28). Kinetically, the contribution of the slowly exchanging cholesterol compartment to the total cellular cholesterol had increased. The increased total cellular cholesterol and phospholipid were a function of the storage granule content of the macrophage, and could be accounted for by the increase in the size of the slowly exchanging cholesterol compartment. These results are consistent with the intracellular cholesterol-rich
membrane localization of the slowly exchanging compartment. The major components of this compartment are the secondary lysosomes largely derived from the plasma membrane. As will be described elsewhere, net synthesis of plasma membrane occurs after extensive endocytosis. Golgi membranes may also make a minor contribution to the intracellular cholesterol compartment under these conditions (14).

The BCG-induced alveolar macrophage and thioglycollate-stimulated peritoneal macrophages represent cells which have increased numbers of lysosomes as a result of in vivo endocytosis. In these larger macrophages exchange proceeded biphasically, and the calculated results showed equal amounts of cholesterol in the rapidly and slowly exchanging compartments. The relative increase in cholesterol was attributable to the increased size of the slowly exchanging compartment and provides further evidence for the plasma membrane and lysosomal membrane localization of the rapidly and slowly exchanging cholesterol pools. The cholesterol attributable to the rapidly exchanging compartment is in close agreement with cholesterol analyses of plasma membrane purified from alveolar macrophages (14, 29).

L-cells and melanoma cells also exchanged their cholesterol with that of serum lipoproteins. These cell types had fewer intracellular cholesterol-rich membranes, and less cholesterol per cell, on the basis of protein. Kinetically, the slowly exchanging compartment was a minor component of the total cell cholesterol. The clear correlation of membrane distribution and cholesterol pool sizes suggests the general validity of the two-compartment model for cellular cholesterol exchange.

From Table III it is clear that the cholesterol content attributable to plasma membrane both per cell and protein is much greater in macrophages than in L-cells and melanoma cells. Although the macrophages may have more surface area, this may not be great enough to account for all the difference in cholesterol. Cholesterol content of plasma membrane may vary for different cell types. A slightly higher membrane cholesterol content could be related to an active role of macrophages in cholesterol metabolism (30) since a high proportion of cholesterol in model membranes favors the entrance of lipid (31).

The data presented here and in the previous paper (1) demonstrate that plasma membrane cholesterol exchanges with that of the intracellular membranes. Exchange takes place in the absence of membrane flow from plasma membrane to lysosomal membrane, and reverse membrane flow is extremely unlikely (16, 17, 20). Since membrane-membrane interactions analogous to soluble lipoproteins-membrane interactions are also unlikely by morphological criteria, an intracellular carrier may exist. Small amounts of cholesterol are found in the soluble fraction of macrophages (1), and sterol carrier proteins have been demonstrative in cholesterol biosynthesis (32, 33). The intracellular concentration of this carrier, its exchangeability, and cholesterol-carrying
capacity could be the rate-limiting step in exchange from the intracellular compartment.

We have described the metabolism of free cholesterol in macrophage membranes. The ingestion, processing, and excretion of cholesterol and cholesterol esters will be discussed in the next communication (30).

SUMMARY

Macrophage membrane cholesterol is present in two subcellular cholesterol pools, a rapidly exchanging compartment comprising about two-thirds of the total cholesterol, and a slowly exchanging compartment comprising one-third of the total. The morphological identification of the kinetically distinguishable pools proceeded by alteration of each compartment. Trypsin treatment markedly decreased the rate of cholesterol exchange without removing cholesterol from the membrane. Recovery of normal exchange rates took more than 7 hr and required protein synthesis. This suggested that a plasma membrane receptor is involved in positioning of lipoproteins for exchange, and is consistent with the plasma membrane localization of the rapidly exchanging compartment.

Extensive pinocytosis by nondegradable dextran, dextran sulfate, or sucrose resulted in the accumulation of many secondary lysosomes, thus increasing the relative proportion of intracellular membranes. The measurable granule membrane area, cholesterol content, phospholipid content, and the relative size of the slowly exchanging cholesterol compartment all increased. The amount of intracellular membrane altered by extensive phagocytosis of latex particles also increased the size of the slowly exchanging cholesterol compartment. This suggested that the slowly exchanging pool of cholesterol represented the intracellular membranes primarily of lysosomal origin.

Rabbit alveolar macrophages and thioglycollate-stimulated peritoneal macrophages contain many secondary lysosomes as a result of multiple bouts of in vivo phagocytosis and pinocytosis. In both of these cells the fast and slow pools are equal in size. The increased cholesterol content was attributable to the increase in the relative size of the slowly exchanging compartment. L-cells and melanoma cells also exchange their cholesterol with that of serum lipoproteins. Both cells contain few cholesterol-rich intracellular membranes, and had lower cellular cholesterol contents. In these cells the slowly exchanging pool was a minor contribution to cell cholesterol. Studies with these cells provided further evidence for the lysosomal membrane and plasma membrane localization of the slowly and rapidly exchanging cholesterol compartments.

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