Previous studies have described the exchange of free cholesterol molecules of macrophage membranes with those of serum lipoproteins (1). Exchangeable cholesterol was found in two compartments. The rapidly exchanging compartment was identified as the plasma membrane, and the slowly exchanging compartment was associated with intracellular membranes (2). Cholesterol esters (CE's) were not exchanged under these conditions, a finding which is in keeping with prior studies employing lipoprotein classes and erythrocytes (3).

Under physiological and pathological conditions macrophages are exposed to other forms of both free cholesterol and CE's. Effete erythrocytes, tissue cells, and chylomicrons are taken up by phagocytosis (4–6), and subsequently are localized within cytoplasmic phagolysosomes. Many of their constituents are then degraded by acid hydrolases (7–9).

Similar events take place under in vitro culture conditions and may lead to the formation of an intralysosomal compartment of free and esterified cholesterol, clearly distinct from the membrane-associated intracellular pool. The processing and fate of cholesterol and CE's within the digestive vacuole is poorly understood.

In this paper we describe the formation of intralysosomal pools of particulate-free and esterified cholesterol, their intracellular processing and excretion from the macrophage, and their influence on the kinetics of exchange. In addition, a lysosomal cholesterol esterase has been characterized and its function evaluated in cell lysates and in the intact macrophage.

**Materials and Methods**

Methods for harvesting and cultivating macrophages, analytic procedures, and microscopy have been described previously (1, 2). For electron microscopy the propylene oxide steps were

*Supported in part by research grants AI 07012 and AI 01831 from the U. S. Public Health Service.

†Current address: Strangeways Research Laboratory, Cambridge, England. (Address reprint requests to The Rockefeller University.)

1Abbreviations used in this paper: BSA, bovine serum albumin; CE, cholesterol ester; CL, cholesteryl linolate; CP, cholesteryl palmitate; EGSS-X, ethylene glycol succinate; GLC, gas-liquid chromatography; NBCS, newborn calf serum; PBS, phosphate-buffered saline; PE, petroleum ether; RBC, red blood cells; TLC, thin-layer chromatography.
omitted and the sample embedded in Epon after 100% alcohol. We are indebted to Doctors James G. Hirsch and Martha E. Fedorko of The Rockefeller University for taking the electron micrographs. All chemicals used were commercially available analytic reagent grades.

Preparation of Cholesterol-14C-Labeled Human Erythrocyte Ghosts.—The washed erythrocytes from 20 ml of fasting venous blood were lysed (10). The plasma was labeled with cholesterol-4-14C (40 mCi/mmole, New England Nuclear Corp., Boston, Mass.) by injecting 10 μCi in 0.5 ml acetone into 5 ml plasma (Millipored) plus 15 ml phosphate-buffered saline (PBS), pH 7.4, containing 20,000 units penicillin. Excess acetone was blown off with a stream of sterile N₂, and the mixture incubated at 37°C on a rocker platform for 20 hr. Approximately 5 X 10⁹ packed ghosts were added to the labeled plasma, 1.45 ml acetone was added, and the mixture was incubated at 37°C for 4 hr (11) and the ghosts centrifuged at 20,000 g for 20 min. The ghosts were washed three times in 20 volumes hypotonic phosphate buffer (20 ideal milliosmolar, pH 7.4), or until washes contained no label. Ghosts contained 3 X 10⁴ disintegrations per minute (dpm) of cholesterol-14C/10⁹ ghosts.

Preparation of Particulate-Free Cholesterol Complexes.—Bovine serum albumin (BSA) (Cohn fraction V, Armour Pharmaceutical Co., Chicago, Ill.) was dissolved in medium 199 to give a 2% (w/v) solution, and sterilized by filtration through a 0.45 μ Millipore filter (Millipore Corporation, Bedford, Mass.). Cholesterol (reagent grade, Mann Research Labs, Inc., New York) was dissolved in acetone at 20 mg/ml. 1 ml of the acetone solution was injected rapidly into 2 ml of the BSA solution. The acetone was removed by a stream of warm N₂, and the resulting mixture was sonicated in an ice bath at setting No. 4 of a Branson Sonifier (Branson Instruments Co., Stamford, Conn.) for 60 sec. The volume of the resulting emulsion (the cholesterol to albumin ratio was 1:2, by weight) was adjusted to 2.0 ml with medium 199, giving a concentration of 10 mg cholesterol/ml. The emulsion consisted of relatively uniform particles about 0.5 μ in diameter and was stable for months. Radioactive complexes were made by adding cholesterol-3H to the cholesterol in acetone solution.

Preparation of Particulate CE Complexes.—CE complexes were made by a method similar to that for the free cholesterol complexes. BSA was used as a 1% solution in medium 199. The CE's were less soluble in acetone, so solutions were made in acetone containing 10-20% petroleum ether (PE) by volume. 2 ml 1% BSA were mixed with 3 ml of acetone solution containing 20 μmoles CE. The organic solvents were blown off with N₂ then processed as above to give 10 μmoles CE/ml. The concentration was checked by gas-liquid chromatography (GLC) after saponification. The resulting complexes (CE/albumin = 1 μmole/mg) were refractile spherules of about 0.5 μ in diameter. Radioactive complexes were made by adding radioactive CE to the unlabeled CE in acetone solution.

Synthesis of Radioactive CE's.—

Plasma CE's: Labeled mixed plasma CE's were prepared by utilizing the lecithin-cholesterol acyltransferase cholesterol esterifying system present in fresh human serum (12). 15 ml of human blood was allowed to clot at 37°C for 4 hr. Serum was recovered after centrifuging at 2000 rpm for 15 min and clarified by repeating the centrifugation step. To 7 ml serum, 70 μCi cholesterol-7α-3H in acetone was added, and the mixture incubated for 16 hr at 37°C on a rocker platform. 14 ml methanol was added to the mixture, and the neutral lipids were extracted with 4-25 ml aliquots of PE. The extracts were pooled, concentrated, and streaked on a preparative thin-layer chromatography (TLC) plate, which was developed in PE-diethyl ether-acetic acid (85:15:1). A small sample was chromatographed on an analytical plate and used to determine radioactivity. About 30% of the label ran with CE's. Spots on the preparative plate were localized with I₂ vapor. The CE band was eluted with PE, and the radioactivity in this band, checked by TLC, was >98% in CE. About 10 mg of CE (10⁶ dpm/mg) were recovered. The CE fatty acids as determined by GLC were 16:0, 8.1%; 61:1, 2.3%; 18:0, 2.7%; 18:1, 19.4%; 18:2, 60.4%.

Cholesteryl-7α-3H linolate (CL) and cholesteryl-7α-3H palmitate (CP): Radioactive CE's
were synthesized on a microscale by reacting 1 mCi (15 Ci/m mole) cholesterol-7α-3H with 20 μl of either linoleoyl chloride (cis,cis,9,12-octadecadienoyl chloride, 99% pure, Analabs Inc., North Haven, Conn.), or palmitoyl chloride (hexadecanoyl chloride, 99% pure, Analabs Inc.) using the method described by Goodman (13). Purity checks by silver nitrate TLC indicated that the final products were >97% CL (15 Ci/m mole) and CP (15 Ci/m mole), respectively.

Enzyme Assays.---

Cholesterol esterase: The reaction mixture in this assay consisted of a small capped tube with 0.1 ml enzyme mixture, 0.4 ml McIlvaine’s citrate-phosphate buffer (14), usually pH 4.0, and 10–100 nmoles CE-3H (usually diluted with “cold” CE to 1 μCi/μmole) blown into the buffer-enzyme mixture in 10 μl acetone. The reaction mixture was incubated at 37°C for 2–10 hr, then the reaction was stopped by adding PE. The radioactive-free and ester cholesterol were extracted with 3–5 ml aliquots of PE, concentrated in a stream of N₂, and an aliquot spotted on a flexible TLC plate (silica gel IB, Bakerflex, J. T. Baker Chemical Co., Phillipsburg, N. J.). Routinely, 10-cm plates were used. The chromatograms were developed in PE-diethyl ether-acetic acid (85:15:1). Cholesterol runs near the origin, while CE’s run near the solvent front in this system. The TLC strip was cut in half and the radioactivity in the two fractions determined. The per cent as free cholesterol compared to the total was the measure of hydrolysis.

Acid phosphatase: This enzyme was assayed at pH 5.0 using sodium acid naphthyl phosphate as substrate (15).

GLC.---Cholesterol was extracted and measured as the trimethylsilyl ether by GLC as described previously (1). Total cholesterol was measured after saponification.

CE fatty acids were determined by GLC on an F and M Biomedical gas chromatograph (Hewlett-Packard Co., Avondale Div., Avondale, Pa.) made available to us in the laboratory of Dr. E. H. Ahrens, Jr. of The Rockefeller University. Fatty acid methyl esters were prepared by transesterification with boron trifluoride-methanol (14% w/v, Applied Science Laboratories Inc., State College, Pa.) by the method of Morrison and Smith (16), and separated on a 6 ft, 4 mm i.d. column of 10% ethylene glycol succinate (EGSS-X) on Gas Chrom P, mesh 100/200 support (Applied Science Laboratories, Inc.), at 182°C column temperature, with N₂ carrier gas, 30–60 ml/min, 20 psi inlet pressure. A hydrogen flame detector was used and the peak sizes were integrated electronically. Fatty acids were identified by comparison with standard fatty acid mixtures (Applied Science Laboratories). The column used had 442 theoretical plates/ft at oleic acid.

Mathematical Methods.---The mathematical analysis of turnover curves used to calculate rate constants was described previously (1).

RESULTS

Ingestion of Cholesterol-14C-Labeled Erythrocyte Ghosts.---Macrophages which had phagocytized antibody-coated erythrocytes contained little morphologically recognizable material 24 hr after ingestion and contained the same amount of cholesterol as control cultures. Erythrocyte membranes, isolated after hypotonic lysis of intact red cells (RBC) were used in this study to increase the efficiency of cholesterol uptake. Human RBC ghosts, labeled by exchange with cholesterol-14C, were coated with opsonizing titers of rabbit–anti-human RBC serum (17). About 12–20 ghosts/macrophage were taken up in 1 hr. The total macrophage cholesterol and phospholipid increased about fourfold. During the subsequent 24 hr period in 20% newborn calf serum (NBCS) morphologically recognizable ghosts disappeared, the excess choles-
terol and phospholipid-phosphorus returned to control levels, and the choles-
terol$^{14}$C counts were recovered in the medium as free cholesterol-$^{14}$C only
(Fig. 1). Thus, cholesterol from within the lysosome was reaching the extra-
cellular milieu.

**Morphological Studies of Ingested Cholesterol-Albumin Complexes.**—The net
flux of cholesterol out of the macrophage was examined in greater detail by
using particulate cholesterol or cholesterol-$^{3}$H-albumin complexes (1:2,
cholesterol:protein, by weight).

Dose response experiments revealed that the complexes were phagocytized
avidly at concentrations of 10–500 μg/ml of cholesterol as complexes, in 20%
After phagocytosis had occurred, the macrophage cultures were rinsed to remove the excess particles, then placed in fresh 20% NBCS. During the next 8 hr, the number of detectable complexes decreased (Fig. 2). Many lipid droplets appeared as a consequence of digestion of the albumin and liberation of the attached free fatty acids. This accumulation of triglyceride also occurred after phagocytosis of heat-denatured albumin particles (Z. Werb, unpublished).

![Fig. 2. Phase-contrast appearance of macrophages after ingestion of particulate cholesterol albumin complexes. Phase contrast X 1250. (a) 15 min after ingestion many irregular, slightly refractile particles are present in the perinuclear zone. The cell has few lipid droplets. (b) 8 hr after ingestion the number of complexes has decreased. The cell has many refractile lipid droplets in a halo at the junction of the cell body and cytoplasmic veil. (c) 48 hr after ingestion this binucleate macrophage has phase-lucent and phase-dense granules, but no recognizable complexes in the centrosomal area. The cell also contains many lipid droplets. (d) A control macrophage containing phase-dense granules and vesicles but few lipid droplets.]

By 48 hr after ingestion, the macrophages contained very few residual complexes (Fig. 2) and were morphologically similar to controls (Fig. 2).

_Ingestion and Intracellular Fate of Cholesterol-3H-Albumin Complexes._—Ingestion of the complexes was linear for at least 6 hr. Up to 150 μg cholesterol was taken up producing a 50–100-fold increase in total cellular cholesterol. In most experiments 10–50 μg of cholesterol was ingested (a 1–4 hr ingestion time). After ingestion the cells were placed in 20% NBCS medium, and the loss of label followed (Fig. 4). The labeled cholesterol was excreted from the macrophages which had ingested the particulate cholesterol complexes, with a single
exponential decay rate. The label recovered in the medium was free cholesterol only. As a control, the washout of cholesterol-$^3$H from macrophages prelabeled by exchange was followed simultaneously. The typical biphasic exchange kinetics were again observed. The cholesterol content of the cholesterol-loaded cells decreased in parallel to the loss of label, with a half-life of 20 hr (Table I). The rate constant describing the relative excretion of cholesterol was $k_c = 0.035$ hr$^{-1}$ (average of five experiments, $k_c = 0.033 \pm 0.004$ hr$^{-1}$); that is, 3.5% of the intralysosomal cholesterol was excreted/hr. The rate constant was invariant over intracellular concentrations of 10-150 $\mu$g cholesterol/flask. The control cells exchanged cholesterol with two exponential phases characterized by half-lives of 3 and 21 hr. The calculated rate constant for the relative movement of cholesterol from the lysosomal membrane to the plasma membrane was $k_{21} = 0.037$ hr$^{-1}$, and the rate constant for movement of cholesterol from the plasma membrane to the medium lipoproteins was $k_{32} = 0.209$ hr$^{-1}$. The

![Ultrastructure of macrophages which have ingested cholesterol-albumin complexes. X 21,000. 30 min after ingestion the cell is filled with irregular phagosomes (arrows) containing complexes arranged in the centrosomal region of the cell. The complexes have an electron-opaque matrix containing electronlucent crystalloids.](image-url)
Fig. 4. Excretion of cholesterol from macrophages after ingestion of cholesterol complexes. 
(a) Ingestion of complexes by macrophages. (b) Loss of label from macrophages after ingestion of cholesterol-albumin complexes. The initial intracellular content of cholesterol, as complexes, was 83 μg/flask. (c) Effect of serum concentration on the rate of cholesterol excretion. The amount of cholesterol ingested as complexes remaining during washout in NBCS medium is shown.

Similar values for $k_s$ and $k_2$ suggested that the rate-limiting reaction for cholesterol excretion from the intralysosomal compartment may occur at the same step as the rate-limiting reaction of the slowly exchanging or lysosomal membrane compartment.

Serum lipoprotein concentration in the medium influenced the rate of cho-
lesterol excretion (Fig. 4). Cholesterol from the intralysosomal particles was lost slowly in 1% NBCS ($k_{c\%} = 0.0087 \text{ hr}^{-1}$), more rapidly in 20% NBCS ($k_{c 20\%} = 0.031$), and most rapidly in 40% NBCS ($k_{c 40\%} = 0.048$). No cholesterol or label was lost from cells incubated in 1% BSA medium. The lipoproteins in the medium also served as the acceptors for the excess cholesterol. In exchange, both the rapid and slowly exponential phases are affected by serum lipoprotein concentration (1).

If cholesterol is excreted from the macrophage by the exchange process,

| Experiment | Time constants | Rate constants |
|------------|---------------|---------------|
| Excretion: | $t_{1/2c}$ | $k_c$ |
| Loss of cholesterol- $^3$H complexes | 20 | 0.035 |
| Exchange: | $t_{1/22}$ | $t_{1/3\phi}$ | $k_{21}$ | $k_{22}$ | $k_{23}$ |
| Loss of cholesterol- $^3$H in washout experiment | 3 | 21 | 0.209 | 0.037 | 0.018 |

* Kinetic constants were determined for washouts in 20% NBCS medium.
† Half-lives are determined directly from graphical analysis.
§ Rate constants $k_{ij}$ represent the rate of motion of cholesterol from one compartment $i$, to another $j$, relative to the mass in compartment $i$ (that is fraction transferred per unit time). $k_{23}$ represents exchange from the plasma membrane (fast) compartment to the medium; $k_{23}$ is the rate from the lysosomal membrane (slow) to the plasma membrane (fast) compartment; $k_{23}$ is the rate for the exchange from the plasma membrane compartment to the lysosomal membrane compartment. For exchange, there is no net flux of cholesterol. For excretion, $k_c ( = \ln(2) / t_{1/2c})$ represents the fraction of the cholesterol of the complexes excreted per unit time.
∥ Value for the experiment described in Fig. 4.b; for five experiments, $t_{1/2} = 21 \pm 3 \text{ hr}$.

now operating to give a net flux of cholesterol, then ingestion of the complexes would change the measured kinetics of exchange. If excretion from intralysosomal pool occurs during external labeling, then a decrease in label taken up over 4 hr would take place. This was observed. Cells filled with nonradioactive cholesterol complexes took up label to 80% of the control value. When labeled medium was withdrawn, and the washout followed, net flux would occur if $k_{23} \rightarrow K_{23} > k_{32}$, or $k_{23} \rightarrow K_{23} < k_{32}$, which would produce net movement of cholesterol from compartment 3 to compartment 2. The consequence of this would be an increased loss of label from the cells. A 50% increase in the rapid rate of loss was observed. Analysis of compartment sizes could not be made for these experiments because a net loss of cholesterol occurred in the
cells containing the complexes, while exchange only occurred in the controls. These results suggest that excretion was occurring via the exchange system.

**Macrophage Acid Cholesterol Esterase.**—Macrophage lysates were tested for the ability to hydrolyze radioactive CE's isolated from serum. Hydrolytic activity with a pH optimum of 4.0 and shoulder towards pH 5 was found (Fig. 5). The reaction was linear for 10 hr (Fig. 5), and obeyed first-order kinetics with respect to enzyme and substrate concentrations. The maximum hydrolysis of the mixed ester substrate was 50%. This may reflect the inherent hydrolyzability of the mixed CE's, or may relate to the physical state of the enzyme and substrate in the reaction mixture. Serum had no cholesterol esterase activity. The hydrolysis of CL was characterized by a sharper maximum rate at pH 4.0 with a shoulder towards the higher pH. CP was hydrolyzed slowly over a broad range of pH with the maximum occurring at pH 5.0.

Intracellular distribution of the cholesterol esterase was determined by differential centrifugation fractionation of macrophages (Table II). Cholesterol esterase activity had the same distribution as acid phosphatase with a twofold enrichment in the large granule fraction. Cholesterol esterase activity was also enriched 2.5-fold in latex phagolysosomes, with 85% of the activity associated with latex-containing fractions. This evidence indicated that the cholesterol esterase was lysosomal.

Cohn (21, 22) reported that both the total and specific activities of acid hydrolases increased during cultivation in high serum medium. A 12-fold
increase in total cholesterol esterase activity using CL as substrate, occurred between 4 and 72 hr of cultivation in 30% NBCS. A fivefold increase in the specific activity occurred to a maximum of 14.2 nmoles CL hydrolyzed/hr per mg protein (Table III).

### TABLE II

| Fraction                        | Protein | Cholesterol esterase | Acid phosphatase |
|---------------------------------|---------|----------------------|------------------|
| Homogenate                      | 815 100 | 0.126 100 0.155   | 98.7 100 121.3  |
| Nuclear (500 g, 10')            | 108 13.3 | 0.027 21.6 0.239  | 13.9 14.0 128.0 |
| Large granule (10,000 g, 30')   | 264 32.5 | 0.087 69.4 0.332  | 79.0 80.0 299.0 |
| Microsomes + soluble (10,000 g, 30') supernatant | 480 58.9 | 0.023 18.3 0.047  | 17.8 18.0 37.1 |
| Recovery                        | 852 104.6 | 137.2 109.3      | 110.7 112.0     |

* Four T-30 flasks, cultivated for 48 hr in 30% NBCS, were used for differential centrifugation fractionation.

† Cholesterol esterase: activity units, nmoles CE (mixed esters) hydrolyzed/min; specific activity (S.A.) units, nmoles/min per mg protein.

§ Acid phosphatase: activity units, nmoles α-naphthol liberated/min; S.A. units, nmoles α-naphthol/min per mg protein.

### TABLE III

| Cultivation in 30% NBCS | CL | CP | Plasma CE's |
|-------------------------|----|----|-------------|
| hr                      | Activity units | S.A. units | Activity units | S.A. units | Activity units | S.A. units |
| 4                       | 0.0033 0.052 | 0.0003 0.0052 | — — –         | 0.0125 0.105 |
| 24                      | 0.0217 0.180 | 0.0005 0.0042 | 0.0250 0.153 |
| 72                      | 0.0383 0.237 | 0.0012 0.072 | — — –         | 0.0125 0.105 |

* Activity units: nmoles CE hydrolyzed/min per T-15 flask.

† Specific activity (S.A.) units: nmoles CE/hr per mg protein; lysates were prepared from T-15 flasks containing 65, 120, and 163 μg cell protein at 4, 24, and 72 hr, respectively.

CL was hydrolyzed much more readily by the macrophage lysates than CP (Table III). With lysates from macrophages cultivated for 72 hr in 30% NBCS the cholesterol esterase specific activity was 33 times as great for CL as for CP. The hydrolysis of the plasma CE's was intermediate in value, per-
haps reflecting differential hydrolysis of the different esters. However, the poor hydrolysis of CP in this assay may in part be due to the physical state of the substrate (solid at 37°C). The biochemical assay was probably a surface reaction with the substrate adsorbed in a layer to the surface of the tube or as a floating layer at the buffer-air interface. Detergents did not facilitate the reaction. Triton X-100 at 5% final concentration, and sodium taurocholate at 4% had no effect. Tween 20 was slightly inhibitory. The presence of PE in the reaction mixture completely inhibited hydrolysis. Iodoacetimide (1 mM) and p-chloromercuribenzoic sulfone (1 mM) inhibited hydrolysis about 50%, while dithiothreitol (1 mM) had no effect.

Rabbit alveolar macrophages also contain cholesterol esterase activity, with a pH optimum of 3.8 and a shoulder towards pH 5.0 when assayed with mixed CE's. At pH 4.0, 0.211 nmoles of mixed serum CE's were hydrolyzed/min per mg protein.

Morphological Studies of Ingested CE's.—CE-albumin complexes were also taken up and sequestered within phagolysosomes. After phagocytosis of CL complexes, phase-dense bodies with refractile inclusions were seen in the perinuclear zone (Fig. 6). Within hours, these complexes became more refractile and decreased in number (Fig. 6 b). By 48 hr few complexes remained (Fig. 6 c), and at this time the cells had about the same number of phase-dense granules as the controls. In the electron microscope, the cells were filled with membrane-bounded CL complexes, with an electron-opaque matrix and more electron-lucent globules (Fig. 7 a). Cells which had ingested CP had a similar phase microscopic appearance immediately after ingestion (Fig. 6 d, e). However, at 24 and 48 hr after phagocytosis (Fig. 6 f), most of the refractile material persisted in the granules arranged in the "hof" of the nucleus.

Ingestion and Intracellular Fate of CL-3H Complexes.—Macrophages were exposed to the complexes in 20% NBCS at a concentration of 0.2 μmoles/ml and uptake was linear for many hours (Fig. 8 b). In 4 hr, each flask had ingested 15.5 nmoles (10 μg) CL. During the subsequent 96 hr in 20% NBCS, label was lost from the cells (Fig. 8 a). Since no cell loss occurred, loss of label was an active process. All the label was recovered in the medium as free cholesterol. The loss of cholesterol from the CL complexes proceeded as a monophasic exponential decay with a half-life of 24 hr (for six experiments t½ = 22 ± 3 hr). The loss was characterized by a rate constant kCL = 0.029 hr⁻¹. Thus, the initial rate of loss of label was 0.029 × 15.5 = 0.45 nmoles/hr per flask (63 pmoles/min per mg cell protein), which was less than the rate of hydrolysis of CL by macrophage lysates in the test tube (Table IV). This suggested that the rate of intracellular hydrolysis of CL was not rate-limiting for excretion. This rate constant was remarkably similar to the rate constant for loss of cholesterol from ingested free cholesterol-albumin complexes, and suggested that a similar rate-limiting step was operating with the ester. When the molecular
Fig. 6. Appearance of macrophages after ingestion of CE complexes. Phase contrast \( \times 1500 \). (a)–(c) CL. (a) 15 min after phagocytosis of complexes numerous phase-dense and refractile granules are present in the perinuclear area. Otherwise the cell is well spread and normal. (b) 5 hr after phagocytosis the granules are more refractile in appearance. (c) 48 hr after phagocytosis few complexes remain. The large refractile objects are triglyceride droplets. (d)–(f) CP. (d) 15 min after phagocytosis of complexes this cell is filled with many irregular masses. (e) 5 hr after phagocytosis little change has occurred. This cell has few large complexes. (f) 48 hr later many granules persisted. Crystals are seen in large phagolysosomes. A few large refractile triglyceride droplets are present outside the granule region.
species of the intracellular label were examined (Table IV) a considerable amount of intracellular free cholesterol was found, and its proportion increased with time. This indicated that the rate of hydrolysis was greater than the rate of excretion. The CL particles contained only CE, and the washout medium contained only free cholesterol. At the start of the washout period, 30% of the ester had already been hydrolyzed intracellularly.

![Image](image)

**Fig. 7.** Ultrastructure of a macrophage which has ingested CL complexes. X 21,000. 30 min after ingestion the cell contains many phagolysosomes (arrows). These membrane-bounded granules contain electron-lucent droplets embedded in an electron-opaque matrix. Other cytoplasmic organelles appear normal.

The intracellular concentration of the CL complexes did not affect the rate of loss of label from the cells (Fig. 8c). Macrophages were incubated in medium containing the complexes for 1.5, 3, and 5 hr. This gave intracellular levels of 10.5, 16.3, and 30 nmoles/flask, respectively, at start of the washout. In all three cases the half-time for loss was 25 hr ($k_{cl} = 0.028$ hr$^{-1}$), which gave initial rates of excretion of cholesterol from the ingested CL complexes of 5.0, 7.7, and 14.0 pmoles/min per flask respectively. In all three cases it was unlikely that the rate of hydrolysis was limiting. Since the number of phagocytic vacuoles was approximately proportional to the amount of particulate material

---

ZENA WERB AND ZANVIL A. COHN

33
Fig. 8. Excretion of CL-3H complexes from macrophages. (a) Loss of label into 20% NBCS from cells which had ingested complexes for 3.5 hr. (b) Uptake of complexes by macrophages from 20% NBCS containing 0.2 μmoles CL/ml in the form of complexes. (c) Effect of initial intracellular concentration of CL on the rate of excretion of label. Initial intracellular levels were 30.0 (○), 16.3 (▲), and 10.5(■) nmoles/flask.

taken up, it is possible that the exponential rate dependence may indicate a constant rate of flux across the lysosomal membrane. This suggests that a constant net flux of cholesterol per unit area of the lysosomal membrane may take place.
Serum lipoprotein concentration of the medium affected the rate of excretion of free cholesterol from the ingested CL complexes (Table V). There was virtually no excretion of label from cells incubated in medium in the absence of serum (1% BSA), although intracellular hydrolysis of cholesterol occurred during the incubation. In this experiment no further increase in rate of loss occurred for concentrations of serum above 20% NBCS. The maximum rate was about 18 nmoles/hr per mg cell protein and occurred in the limiting case where the rate of excretion of cholesterol was equal to or greater than the rate of hydrolysis of CL.

For the usual intracellular concentrations of phagocytized CL complexes,
in 20% NBCS, the cholesterol esterase was not limiting and the rate of excretion of free cholesterol was the rate-determining reaction.

**Intracellular Fate of Ingested CP-3H complexes.**—CP-albumin complexes were phagocytized as readily by macrophages as CL-albumin complexes. However, cholesterol from CP complexes was lost very slowly (Fig. 9). Starting with initial intracellular concentrations of 15 nmoles in both cases, the rate of loss of label from cells ingesting CP was \( k_{CP} = 0.01 \text{ hr}^{-1} \), compared to rate of loss from cells ingesting CL of \( k_{CL} = 0.028 \text{ hr}^{-1} \). In other experiments \( k_{CP} \) varied from 0.0115 to 0.006 \text{ hr}^{-1} \) for initial intracellular concentrations of 10–27 nmoles CP/flask. Unlike the CL-filled cells, the amount of radioactive free cholesterol was small and did not change with time in the CP-filled cells (Table IV).

Increase or decrease of serum lipoprotein concentration present in the extracellular fluid had little effect on excretion of label from cells ingesting CP. In the absence of lipoproteins (1% BSA medium) no label was excreted.

This data indicated a slower rate of intracellular hydrolysis of CP. The rate-limiting reaction for excretion of cholesterol from cells ingesting CP complexes was the rate of hydrolysis.
Intracellular Fate of Mixed Serum CE's.—Cholesterol-³H esters labeled by the lecithin-cholesterol acyltransferase reaction in fresh human serum were also used to study intracellular processing of CE in macrophages. The major component of the serum esters was CL (60.4%). However, considerable quantities of cholesteryl oleate (19.4%), CP (8.1%), and others eters (12.1%) were also present. The mixed CE-albumin complexes were taken up avidly and resulted in similar morphological alterations. During the subsequent incubation in 20% NBCS, label was lost from the cells, and recovered in the medium as free cholesterol (Fig. 10). A biphasic or multiphasic exponential loss curve was seen, in contrast to the single exponential loss of label in the case of a single molecular species of CE. The shape of the excretion curve suggested differential hydrolysis of the different CE's in the mixture.

DISCUSSION

Fate of Free Cholesterol.—The results indicate that phagocytosis of cholesterol leads to the net accumulation of cell cholesterol which is then excreted into the medium. In contrast cholesterol exchange does not result in the net accumulation of cholesterol (1). Excretion of cholesterol probably occurs by the same mechanism as exchange and does not involve bulk exocytosis (22, 23).
Particulate cholesterol is present intralysosomally surrounded by the cholesterol-rich lysosomal membrane comprising the cellular, slowly exchanging pool. Initially, all the label is present within this intralysosomal compartment and only a single exponential decay rate is seen. The more rapid rates in the sequence may be “hidden” behind the rate-limiting reaction (24). This decay is described by

\[ C^* = C_0^* e^{-k_e t} \]

where \( C^* \) is the amount of cholesterol (or label) remaining, \( C_0^* \) is the initial amount of cholesterol (or label), \( t \) is the time, and \( k_e \) is the rate constant = \( \ln 2 / t_{1/2} \). \( k_e \) describes the rate-limiting step, and indicates that about 3% of the cholesterol remaining is excreted/hr. Although the precise location of the rate-limiting step is unknown, the value of \( k_e \) is invariant over a wide range of intracellular cholesterol concentrations (10–150 \( \mu \)g cholesterol/flask at time zero). The complexes are taken up into many phagolysosomes thereby increasing the area of lysosomal membrane surface. Exchange probably occurs between the cholesterol at the surface of the complexes and the lysosomal membrane cholesterol. Since the membrane surface area increases proportionately to the number of the particulate complexes ingested, the cholesterol flux across each unit area of membrane may be constant. Initially there are many phagolysosomes and the available lysosomal membrane area is large. As the complexes are modified intralysosomally the size and number of secondary lysosomes decrease, resulting in a decreased lysosomal membrane area across which cholesterol excretion can occur. Since the value of the rate constant \( k_2 \) describing the relative movement of cholesterol from the lysosomal membrane to the plasma membrane during exchange is similar in value to \( k_e \), the rate-limiting reaction probably occurs at the same step for both exchange and excretion. In the absence of membrane apposition or translocation, an intracellular soluble “carrier” may mediate the transfer of cholesterol between the lysosomal membrane and the plasma membrane (2). Cholesterol may therefore move from within the lysosome to the lysosomal membrane readily, whereas the transfer to the intracellular carrier, or its concentration may be rate limiting.

Cholesterol exchange kinetics are affected by the presence of intralysosomal cholesterol. The uptake of label by exchange was depressed about 20%. Since the concentration of cholesterol per unit membrane is probably constant (18), this observation suggests that during excretion, net flux of cholesterol out of the macrophage results in the reduced capacity of the membrane to accept cholesterol from the serum lipoproteins. The presence of intralysosomal cholesterol accelerated the rate of washout of label from macrophages which have membrane cholesterol labeled by exchange. This suggests that during cholesterol excretion membrane cholesterol is displaced by cholesterol excreted from the lysosome, while the membrane cholesterol remains constant.
The plasma membrane cholesterol compartment may become labeled during cholesterol excretion; however the rapid rate of transfer is hidden behind slower rates in the sequence. The serum lipoprotein dependency of both excretion and exchange suggests that cholesterol translocation in the macrophage involves membrane-lipoprotein reactions at the plasma membrane.

**Fate of Cholesterol Esters.**—CE's as particulate albumin coacervates are taken up by macrophages and hydrolyzed by a lysosomal CE hydrolase. This enzyme has a pH optimum of 4.0 with unsaturated CE's, and 5.5 with CP. CL is hydrolyzed much more readily than CP both in the test tube and intralysosomally. Unlike pancreatic cholesterol esterase (25), bile acids and detergents are not required for activity. A cholesterol esterase active at acid pH has not been demonstrated clearly in other tissues (6, 8, 26-28). An exception is the report of Patrick and Lake (29) that an acid "lipase" capable of hydrolyzing both triolein and cholesteryl oleate at pH 4.6 is absent in Wolman's disease, an inborn error in which both triglycerides and CE's accumulate within storage granules. No cholesterol esterifying activity was found in the macrophage, in contrast to observations of Day (30-32).

CE's are not exchanged. In the macrophage unhydrolyzed esters remain intralysosomally. This is in keeping with observations that CE's do not exchange between lipoprotein classes in plasma (3), and that little esterified cholesterol is returned to the medium of LS178Y mouse lymphoblasts which contain considerable levels of CE's (33-35). Excretion of free cholesterol, liberated by the hydrolysis of CE's, occurs by the same mechanism as excretion of excess free cholesterol from ingested free cholesterol complexes. The requirement for serum lipoprotein, the characteristic rate constant, and the effect on exchange kinetics suggest that cholesterol excretion utilizes the same "transport" system as exchange of cholesterol. This provides a mechanism for clearing lysosomes of free and esterified cholesterol brought in by bulk transport.

The intralysosomal hydrolysis of CE's has many implications for lipid processing by cells. The active cholesterol esterase present in the macrophage may result in the absence of detectable CE in these cells. The CE content of other cells cultivated in vitro (35) may indicate low levels of intralysosomal CE's, so that pinocytized CE's are not hydrolyzed and accumulate intralysosomally.

**A General Model for Cholesterol Metabolism in the Macrophage.**—The scheme proposed in Fig. 1 accounts for many of our observations on membrane-mediated cholesterol exchange (1, 2) and excretion. This leads to some generalizations about the functions of mononuclear phagocytes in cholesterol metabolism. Only bulk transport results in macrophage storage of cholesterol in vitro. In vivo, effete RBC, dead cells, and polymorphonuclear leukocytes from inflammatory sites may serve as cholesterol sources. Chylomicron CE's are taken up by Kupffer's cells. Turnover of cell constituents by autophagy (36-38) may
also produce intralysosomal cholesterol. This cholesterol would be transferred to soluble lipoproteins in the tissue spaces. Excretion of cholesterol may be efficient where the soluble lipoprotein concentration is high, and less efficient at low concentrations of lipoprotein.

There are a number of disorders of lipid metabolism in which circulating and tissue macrophages are implicated. Alteration of lysosomal hydrolases results in the storage of nondigested molecules segregated by autophagy and heterophagy (39) and occurs for CE's in Wolman's disease. However, there are a number of storage diseases in which cholesterol accumulates in tissues, although
the lesion is not known to involve cholesterol metabolism. We have demonstrated that net increases in both cholesterol and phospholipid can be considerable in cells containing storage granules, the contents of which are free of lipid (2). These increases are due to the membrane of the secondary lysosomes. Thus in Tay-Sachs (40, 41) and Niemann-Pick diseases (42) the increases in cholesterol may be the consequence of increased lysosomal membrane surrounding the stored lipid.

In xanthomas, macrophages may be phagocytizing lipid of chylomicron origin (43, 44) more rapidly than it can be excreted by the membrane-mediated transport system. This may also be the case for macrophage foam cells in atherosclerotic plaques since much of the lipid in the macrophages is membrane bounded (45, 46). Lower concentrations or impaired cholesterol-carrying capacities of extracellular lipoproteins may also result in intracellular accumulation of cholesterol and CE's in these diseases.

SUMMARY

Phagocytosis of cholesterol-containing particles resulted in the formation of an intralysosomal cholesterol compartment. Cholesterol was excreted out of the macrophage with a single exponential rate which depended on the concentration of acceptor lipoproteins in the medium. Exchange kinetics performed on cells which had ingested particulate cholesterol suggested that excretion occurred by the same mechanism as exchange.

Cholesterol esters as particulate albumin coacervates were taken up by macrophages and hydrolyzed by a lysosomal cholesterol esterase with optimal activity at pH 4.0. Cholesteryl linoleate was hydrolyzed much more readily than cholesteryl palmitate. The amount of cholesterol esterase and its specific activity increased during the in vitro cultivation of macrophages. Intralysosomally, cholesteryl linoleate and palmitate were hydrolyzed to free cholesterol which was excreted from the macrophage and recovered in the medium. Since cholesteryl linoleate was hydrolyzed more rapidly than free cholesterol was excreted into the medium, free cholesterol accumulated intralysosomally. Cholesteryl palmitate was hydrolyzed more slowly, and the rate of hydrolysis was limiting for excretion of the free cholesterol from within the lysosome.

BIBLIOGRAPHY

1. Werb, Z., and Z. A. Cohn. 1971. Cholesterol metabolism in the macrophage. I. The regulation of cholesterol exchange. J. Exp. Med. 134:1545.
2. Werb, Z., and Z. A. Cohn. 1971. Cholesterol metabolism in the macrophage. II. Alteration of subcellular cholesterol compartments and exchange in other cell types. J. Exp. Med. 134:1570.
3. Roheim, P. S., D. E. Haft, L. E. Gidez, A. White, and H. A. Eder. 1963. Plasma lipoprotein metabolism in perfused rat livers. II. Transfer of free and esterified cholesterol into the plasma. J. Clin. Invest. 42:1277.
MACROPHAGE PROCESSING OF CHOLESTEROL AND ITS ESTERS

4. Florey, H. 1962. General Pathology. W. B. Saunders Company, Philadelphia. 3rd edition.
5. Byers, S. O. 1960. Lipids and the reticuloendothelial system. *Ann. N. Y. Acad. Sci.* 88:240.
6. Stein, O., Y. Stein, D. S. Goodman, and N. H. Fidge. 1969. The metabolism of chylomicron cholesteryl ester in rat liver. A combined radioautographic, electron microscopic, and biochemical study. *J. Cell Biol.* 43:410.
7. Cohn, Z. A., and E. Wiener. 1963. The particulate hydrolases of macrophages. I. Comparative enzymology, isolation and properties. *J. Exp. Med.* 118:991.
8. Fowler, S., and C. de Duve. 1969. Digestive activity of lysosomes. III. The digestion of lipids by extracts of rat liver lysosomes. *J. Biol. Chem.* 244:671.
9. Barrett, A. J. 1969. Properties of lysosomal enzymes. In *Lysosomes in Biology and Pathology*. J. T. Diingle and H. B. Fell, editors. North Holland Publishing Company, Amsterdam. 2:245.
10. Dodge, J. T., C. Mitchell, and D. S. Hanahan. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* 100:119.
11. Bruckdorfer, K. R., J. M. Graham, and C. Green. 1968. The incorporation of steroid molecules into lecithin sols, β-lipoproteins and cellular membranes. *Eur. J. Biochem.* 4:512.
12. Glomset, J. A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res.* 9:155.
13. Goodman, D. S. 1969. Hydrolysis and formation of cholesterol esters in rat liver. In *Methods in Enzymology*. Steroids and Terpenoids. R. B. Clayton, editor. Academic Press, Inc., New York. 15:522.
14. Sober, H. A. 1968. Selected data for molecular biology. In *Handbook of Biochemistry*. Chemical Rubber Co., Cleveland, Ohio. J-195.
15. Axline, S. G. 1968. Isozymes of acid phosphatase in normal and Calmette-Guérin bacillus-induced rabbit alveolar macrophages. *J. Exp. Med.* 128:1031.
16. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J. Lipid Res.* 5:696.
17. Rabinovitch, M. 1970. Phagocytic recognition. In *Mononuclear Phagocytes*. R. van Furth, editor. Blackwell Scientific Publications, Ltd., Oxford, England. 299.
18. Werb, Z. 1971. Dynamics of macrophage membrane cholesterol. Ph.D. Thesis. The Rockefeller University, New York.
19. Reiner, J. M. 1953. The study of metabolic turnover rates by means of isotopic tracers. I. Fundamental relations. *Arch. Biochem. Biophys.* 46:53.
20. Reiner, J. M. 1953. The study of metabolic turnover rates by means of isotopic tracers. II. Turnover in a simple reaction system. *Arch. Biochem. Biophys.* 46:80.
21. Cohn, Z. A. 1966. The regulation of pinocytosis in mouse macrophages. I. Metabolic requirements as defined by the use of inhibitors. *J. Exp. Med.* 124:557.
22. Cohn, Z. A. 1968. The structure and function of monocytes and macrophages. *Adv. Immunol.* 9:163.
23. Cohn, Z. A., and B. Benson. 1965. The in vitro differentiation of mononuclear phagocytes. III. The reversibility of granule and hydrolytic enzyme formation and the turnover of granule constituents. *J. Exp. Med.* 122:455.
24. Solomon, A. K. 1960. Compartmental methods of kinetic analysis. In Mineral Metabolism, An Advanced Treatise. Principles, Processes and Systems, part A. C. L. Comar and F. Bronner, editors. Academic Press, Inc., New York. 1:118.
25. Wilson, J. D., and R. T. Reinke. 1968. Transfer of locally synthesized cholesterol from intestinal wall to intestinal lymph. J. Lipid Res. 9:85.
26. Deykin, J. M., and D. S. Goodman. 1962. Hydrolysis of long-chain fatty acid esters of cholesterol with rat liver enzymes. J. Biol. Chem. 237:3649.
27. Stoffel, W., and H. Greten. 1967. Studies on lipolytic activities of rat liver lysosomes. Hoppe-Seyler’s Z. Physiol. Chem. 348:1145.
28. Day, A. J. 1960. Cholesterol esterase activity of rabbit macrophages. Quart. J. Exp. Physiol. Cog. Med. Sci. 45:55.
29. Patrick, A. D., and B. D. Lake. 1969. Deficiency of an acid lipase in Wolman’s disease. Nature (London). 222:1067.
30. Day, A. J. 1967. Lipid metabolism by macrophages and its relation to atherosclerosis. Advan. Lipid Res. 5:185.
31. Day, A. J., and P. R. S. Gould-Hurst. 1963. The effect of lecithin on cholesterol esterase activity of macrophages. Aust. J. Exp. Biol. Med. Sci. 41:823.
32. Day, A. J., and R. K. Tume. 1969. Cholesterol-esterfying activity of cell-free preparations of rabbit peritoneal macrophages. Biochim. Biophys. Acta. 176:367.
33. Rothblat, G. H., R. Hartzell, H. Mialhe, and D. Kritchevsky. 1967. Cholesterol metabolism in tissue culture cells. Lipid Metab. Tissue Cult. Cells. Wistar Inst. Symp. Monogr. 6:129.
34. Rothblat, G. H. 1969. Lipid metabolism in tissue culture cells. Advan. Lipid Res. 7:135.
35. Rothblat, G. H., and D. Kritchevsky. 1968. The metabolism of free and esterified cholesterol in tissue culture cells: a review. Exp. Mol. Pathol. 8:314.
36. Fedorko, M. E., J. G. Hirsch, and Z. A. Cohn. 1968. Autophagic vacuoles produced in vitro. I. Studies on cultured macrophages exposed to chloroquine. J. Cell Biol. 38:373.
37. Fedorko, M. E., J. G. Hirsch, and Z. A. Cohn. 1968. Autophagic vacuoles produced in vitro. II. Studies on the mechanism of formation of autophagic vacuoles produced by chloroquine. J. Cell Biol. 38:392.
38. Hirsch, J. G., M. E. Fedorko, and Z. A. Cohn. 1968. Vesicle fusion and formation at the surface of pinocytic vacuoles in macrophages. J. Cell Biol. 38:629.
39. Hers, H. G., and F. van Hoof. 1969. Genetic abnormalities of lysosomes. In Lysosomes in Biology and Pathology. J. T. Dingle and H. B. Fell, editors. North Holland Publishing Company, Amsterdam. 2:19.
40. Okada, S., and J. S. O'Brien. 1969. Tay-Sach’s disease: generalized absence of a beta-N-acetylhexosaminidase component. Science (Washington). 165:998.
41. Brady, R. O. 1970. Cerebral lipidoses. Annu. Rev. Med. 21:317.
42. Fredrickson, D. S. 1965. Sphingomyelin lipidosis: Niemann-Pick Disease. In The Metabolic Basis of Inherited Disease. V. B. Stanbury, V. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Company, New York. 2nd edition. 586.
43. Parker, F., and G. F. Odland. 1968. Experimental xanthoma. A correlative biochemical, histologic, histochemical and electron microscopic study. Amer. J. Pathol. 53:537.
44. Parker, F., J. D. Bagdade, G. F. Odland, and E. L. Bierman. 1970. Evidence for the chylomicron origin of lipids accumulating in diabetic eruptive xanthoma: a correlative lipid biochemical, histochemical and electron microscopic study. J. Clin. Invest. 49:2172.

45. Geer, J. C. 1965. Fine structure of canine experimental atherosclerosis. Amer. J. Pathol. 47:242.

46. Moss, N. S., and E. P. Benditt. 1970. The ultrastructure of spontaneous and experimentally induced arterial lesions. III. The cholesterol-induced lesions and the effect of a cholesterol and oil diet on preexisting spontaneous plaque in chicken aorta. Lab. Invest. 23:521.