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CHOLESTEROL METABOLISM IN THE MACROPHAGE

I. The Regulation of Cholesterol Exchange*

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Cholesterol is a constituent of all mammalian cells and serves as a structural unit of membranes. Cytomembranes of the "thick" variety (80–100 A) which have potential access to the extracellular milieu are rich in cholesterol and include plasma membrane, lysosomal membrane, and secretion granules (1–10). In contrast, "thin" membranes (40–70 A) such as endoplasmic reticulum, mitochondria, and nuclear envelope are poor in cholesterol, whereas Golgi membranes are intermediate in size and cholesterol content (5, 11, 12).

Although there is an extensive literature on cholesterol metabolism of the whole organism (13–16), our knowledge of the cellular level is fragmentary. Exceptions are the well-documented studies first described by Hagerman and Gould (17) demonstrating cholesterol exchange from plasma lipoproteins to the membrane of erythrocytes. No net change in cholesterol content of red cell takes place and cholesterol ester (CE)[1] exchange is very limited. Recently, the *de novo* biosynthesis of cholesterol by cultured eukaryotic cells has been shown (18–20) although its conversion to metabolites other than its esters has not been demonstrated (19, 21, 22).

We have chosen to examine the metabolism of cholesterol in homogeneous populations of macrophages. This was prompted by the physiological role of this cell in the processing of chylomicrons and effete erythrocytes (23–25) and its association with a number of pathological events in which lipid storage takes place (26–30). In addition, macrophages exhibit striking endocytic activity in which the flow and turnover of cholesterol-rich membranes might be examined.

In this article we will describe the content and subcellular distribution of macrophage cholesterol, the factors controlling exchange, and its biosynthesis under defined cultural conditions.

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CE, cholesterol ester; FCS, fetal calf serum; GLC, gas-liquid chromatography; NBCS, newborn calf serum; PBS, phosphate-buffered saline; PE, petroleum ether; TC 199, medium 199; TLC, thin-layer chromatography.

Materials and Methods

Harvesting and Cultivation of Mouse Peritoneal Macrophages.—Unstimulated mouse peritoneal macrophages were obtained from NCS mice as described previously (31, 32). Cells were cultivated on flying cover slips for morphological observations, and in 15 or 30 cm^2 T-flasks for biochemical studies. In most experiments, the macrophages were cultivated at 37°C, in 20% newborn calf serum (NBCS, Grand Island Biological Co., Grand Island, N.Y.) in medium 199 (TC 199, Microbiological Associates, Inc., Bethesda, Md.).

Cholesterol-7α-^3H.—Benzene was removed by a stream of warm N_2 from the cholesterol-7α-^3H (15 Ci/mmole, New England Nuclear Corp., Boston, Mass.), then dissolved in acetone at 0.5 mCi/ml, and stored at 4°C under N_2. Purity checks by thin-layer chromatography (TLC) showed that the label was greater than 98% in cholesterol.

Labeling of Serum Lipoproteins.—Cholesterol-^3H in acetone was injected rapidly into serum, or serum mixed with TC 199, then allowed to equilibrate at 37°C with rocking for 1–25 hr. The trace amounts of acetone (usually 1 μl/ml medium) did not affect the cells. The final medium generally contained 1 μCi/ml in 10% fetal calf serum (FCS) or 20% NBCS. This is equivalent to specific activities of 1 μCi/0.1 μmole free cholesterol and 1 μCi/μmole free cholesterol, respectively, for the lot numbers of serum used for more than 80% of the experiments. These sera contained no detectable lecithin-cholesterol acyltransferase activity (33).

"Uptake" Experiments.—A standard procedure was employed for studying exchange of cholesterol by influx of label. Macrophages cultivated for 24 hr in 20% NBCS were labeled by exposure to cholesterol-^3H containing serum medium (either 10% FCS or 20% NBCS). After a given time of exposure, the medium was aspirated and the cell sheet washed gently, four times, with 5-ml saline washes for T-15 flasks. The efficacy of the washing procedure was monitored and four washes were adequate to remove all label from cells exposed for 15 sec or less at room temperature. Cells and protein were not lost for up to eight washes. Macrophages were then prepared for analytic procedures by adding 1.0 ml saline and disrupted by six cycles of freezing and thawing.

"Washout" Experiments.—Cells were exposed to radioactive medium for a given pulse time. The medium was aspirated and the cell sheet washed gently four times with TC 199. This procedure adequately removed the adsorbed label without disturbing the cells. Fresh medium was added and incubation continued. At the end of the washout, the medium was aspirated, the cell sheet washed four times with saline, and harvested in 1.0 ml saline.

Preparation of Cells for Radioactivity Measurements.—To assay total radioactivity 0.1 ml cell preparation was mixed with 1.0 ml NCS solubilizer (Nuclear-Chicago, Des Plaines, Ill.) and 10 ml Liquifluor-toluene (New England Nuclear Corp.) was added. In the case of cholesterol-^3H, all the label was soluble in lipid solvents and extraction was unnecessary. All samples of ^3H and ^{14}C were counted in a Nuclear-Chicago Mark II liquid scintillation spectrometer. Counting rates were corrected for quenching with an external standard, and are expressed in disintegrations per minute (dpm). Samples of thin-layer chromatograms, polyacrylamide gels, and paper chromatograms were expressed as counts per minute (cpm).

Cell Fractionation by Differential Centrifugation.—Macrophage homogenates were separated into crude fractions by differential centrifugation. Macrophages in T-30 flasks (usually four to six flasks) were rinsed with four 10-ml washes of medium 199, scraped with a rubber policeman into a total of 5 ml, 8.5% (w/v) sucrose, and homogenized in a 7 ml Dounce homogenizer (Kontes Glass Co., Vineland, N.J.). About 15 strokes were required to produce >90% cell rupture, as monitored by phase microscopy. An aliquot of the homogenate was set aside, and the rest centrifuged at 500 *g* for 10 min in a 9 RA angle-head rotor (Lourdes Instrument Corp., Old Bethpage, N.Y.). The supernatant was decanted and centrifuged again at 10,000 *g*

for 30 min. The pellet (nuclear fraction) was resuspended in 1.0 ml, 8.5% sucrose. The supernatant was decanted and centrifuged in the SW39 rotor (Beckman L-2, Beckman Instruments, Inc., Fullerton, Calif.) at 100,000 *g* for 60 min. The pellet (large granule fraction) was resuspended in 1.0 ml of 8.5% sucrose. At the end of the ultracentrifugation the floating lipid pellicle was carefully removed, and the soluble fraction decanted. The final translucent pellet (microsomal fraction) was resuspended in 1.0 ml 8.5% sucrose and all fractions rapidly frozen and thawed six times before assaying lysosomal enzymes.

Extraction of Lipids.—Total lipids were extracted with chloroform-methanol by the method of Folch et al. (34). Neutral lipids, including cholesterol esters were quantitatively extracted from aqueous mixtures such as cell lysates by mixing 1 volume with 3 volumes of methanol, followed by 15 volumes of low-boiling petroleum ether (PE). The two phases were mixed vigorously by vortexing, and the PE phase (upper) was removed. The PE extraction was repeated twice more using 10 volumes PE.

Thin-Layer Chromatography (TLC).—Neutral lipids were separated on silica gel TLC plates (Silica gel 1-B, Baker-Flex, J. T. Baker Chemical Co., Phillipsburg, N.J.) which had been activated at 100°C for 20 min. Lipid extracts were placed on the plates and separated by ascending chromatography in PE:diethyl ether:glacial acetic acid (85:15:1) (35). For assaying radioactivity, the chromatograms were cut into 1-cm strips, placed in scintillation vials, and toluene scintillation fluid was added. Nonradioactive standards, cochromatographed to serve as markers, were detected using dichlorofluorescein spray (Applied Science Laboratories, Inc., State College, Pa.), and visualized with UV light. Preparative TLC plates (0.25 mm thick) were prepared using Silicic Acid H (Brinkmann Instruments, Westbury, N.Y.) and were activated before use for 60 min at 110°C. Neutral lipids were separated as above. Spots were marked after exposure to dichlorofluorescein or iodine vapor, scraped from the plates, and neutral lipids eluted with PE.

Gas-Liquid Chromatography (GLC) of Sterols.—Cholesterol was extracted with petroleum ether as outlined above, with the addition of 5α-cholestane (25 μl of 56 μg/ml in ethyl acetate for every 1–5 μg cholesterol expected in the sample) as an internal standard before extraction. The extract was taken to dryness under N_2, and the trimethylsilyl ethers prepared (36) by adding 25 μl of trimethylsilizane in pyridine (Sil-Prep, Applied Science Laboratories, Inc.) and incubating for at least 30 min at room temperature. The mixture was separated on a 4 ft, 4 mm I.D. column of 1% DC 560 (chlorophenylmethyl silicone) on Gas Chrom P at 215°C column temperature, performed on an F and M Biomedical Gas Chromatograph, model 400 (F and M Scientific Corp., Hewlett-Packard Co., Avondale Div., Avondale, Pa.). We are indebted to Dr. E. H. Ahrens, Jr. of The Rockefeller University for the use of his GLC laboratory. The column had a separation capacity of 463 theoretical plates/ft at cholesterol. The hydrogen flame detector had a linear response achieved over 0.02 to at least 120 μg of sample injected. Peak sizes were integrated automatically. Retention times relative to 5α-cholestane were 2.24 for cholesterol, 2.48 for $\Delta^{5,7}$ cholesterol, 2.50 for desmosterol, and 3.65 for β-sitosterol (lipid standards were purchased from Applied Science Laboratories, Inc.). The amount of sterol present was determined by comparing peak area to that of the known internal standard, 5α-cholestane. The peak sizes were directly proportional to the actual weights of the unsubstituted parent compounds. Generally, one-fifth to one-half of the cells of a T-15 flask, or about 0.5 to 1 $\times$ 10^6 cells, were extracted. When compared with 1.4 μg 5α-cholestane standard, this number of cells contained about 1 μg cholesterol. The aliquot injected onto the column contained about 0.1 μg cholesterol.

The procedure described above measured only free cholesterol. To determine total cholesterol or cholesterol esters, the esters were first saponified to the free sterol. The sample plus internal standard (5α-cholestane) were placed in a tube with a tight-fitting cap. 2 ml 0.5 N NaOH in 90% ethanol were added, and refluxed for 60 min in an 80°C sand bath. Saponifica-

tion was checked by TLC. The tube was cooled, 1 ml water was added, and free sterol extracted with three 5-ml aliquots of petroleum ether (low boiling). The extracts were taken to dryness, then prepared for GLC by making the trimethylsilyl derivatives as described above.

Electrophoresis of Serum Lipoproteins.—Paper electrophoresis of serum lipoproteins labeled by equilibration with cholesterol-^{3}H was performed for us by Dr. R. S. Lees, using the procedure of Hatch and Lees (37).

Gel electrophoresis was performed on 6 cm, 5% polyacrylamide gels, pH 9.5 (38). Cholesterol-^{3}H-labeled serum samples (10 μl) were mixed with 40 μl 20% sucrose and layered on the gel. In some samples lipoproteins were prestained by mixing two volumes serum with one volume saturated Sudan Black B in ethylene glycol (39–41). The gels were electrophoresed using 2.5 ma/tube for 40 min. For radioactivity measurements the gels were frozen on dry ice and sliced into 1 mm pieces. 0.5 ml NCS solubilizer was added to each aliquot, and toluene-Liquifluor scintillation fluid added. For protein staining, gels were fixed in 12.5% trichloroacetic acid (TCA) for 30 min, stained in Coomassie blue (1% aqueous, diluted 1/20 with 12.5% TCA) for 30 min, then destained overnight and stored in 10% TCA. Gels prestained with Sudan Black B were fixed in 12.5% TCA and examined immediately, since the stain was unstable. Good resolution was obtained although varying amounts of protein did not penetrate into the gels.

Acid phosphatase assays were carried out using β-glycerophosphate (15.3 mg/ml) (31), or using α-naphthol acid phosphate (0.01 M) (42) as substrate at pH 5.0.

5′ nucleotidase was assayed at pH 8.5 using 5′ adenosine monophosphate (AMP) (43).

Protein was determined by the method of Lowry et al. (44).

Other Materials.—Serum protein fractions, Pentex Biochemical, Kankakee, Ill.; sodium β-glycerophosphate, Eastman Kodak Co., Rochester, N.Y.; sodium α-naphthol acid phosphate, Dajac Laboratories, Philadelphia, Pa.; sodium acetate-1-^{14}C (22.3 mCi/mole), New England Nuclear Corp. All lipids were purchased from Applied Science Laboratories, Inc.; all other chemicals were commercially available, analytic reagent grade.

RESULTS

Cholesterol and Cholesterol Ester Content of Macrophages.—Initial experiments were performed to determine the nature and quantity of macrophage sterols. More than 99% of cell sterol is present as cholesterol and occasionally a trace peak corresponding to desmosterol could be detected.

Macrophages cultivated in 1–30% NBCS increase in size and protein content with 1–72 hr in culture. Free cholesterol increases in parallel to the protein content, and the cholesterol to protein ratio remains remarkably constant at 1.2 $\pm$ 0.18 μg cholesterol/100 μg protein. Cells grown in 40–50% NBCS have a slightly higher cholesterol content, 1.4 $\pm$ 0.2 μg cholesterol/100 μg cell protein. These cells have many secondary lysosomes formed as the result of extensive pinocytosis (45).

Cholesterol esters were never detected in washed mouse macrophages. Saponification of the cholesterol extracts before preparation for GLC results in no increment in the cholesterol measured. When Folch extracts of macrophages were separated by TLC, and the areas corresponding to free cholesterol and cholesterol esters eluted and prepared for GLC, no detectable cholesterol ester was found, even at concentrations where 1% of the total cholesterol as ester could be determined accurately. In at least 30 TLC runs of extracts of macro-

phages labeled with cholesterol-^{3}H, no radioactive cholesterol ester was detected. The constant cholesterol/protein ratios provided a rapid and easy method of describing specific activities of radioactive experiments on the basis of protein and where necessary were used to normalize cultures varying in cell number.

The Distribution of Cholesterol in Subcellular Fractions.—Fractions separated by differential centrifugation were monitored by high resolution phase microscopy and enzymatic markers. Table I shows the results of a fractionation of macrophages labeled for 5 hr in 20% NBCS containing 1 μCi cholesterol-^{3}H/ml. Most of the cholesterol was recovered in nuclear, large granule, and microsomal fractions. The large granule fraction contained most of the cholesterol-rich lysosomes, as judged by acid phosphatase recovery. The nuclear and microsomal fractions contained most of the plasma membrane as judged by 5′ nucleotidase recovery. The specific activity of cholesterol was virtually identical in all fractions. The small amounts of cholesterol in the nuclear, soluble, and lipid pellicle fractions made their specific activity uncertain. Only a trace amount of cholesterol was found in lipid droplets. After a second 100,000 *g*, 60 min centrifugation, less than 3% of the cholesterol-^{3}H was recovered in the soluble portion.

TABLE I

Distribution of Cholesterol-^{3}H in Subcellular Fractions

Fraction	Cholesterol					Protein		Acid phosphatase		
	μg*	%*	*dpm* $\times 10^{-5}$	%	*S.A.* dpm/μg* $\times 10^{-5}$	μg	%	*nmoles α-naphthol/min*	%	*nmoles α-naphthol/min per mg protein*
Homogenate	29.0	100	34.6	100	1.20	1440	100	76.6	100	53.3
Nuclear (500 *g*, 10 min, pellet)	(3.6)	(9.0)	6.67	19.3	(1.86)	426	29.6	14.4	18.8	33.7
Large granule (10,000 *g*, 30 min, pellet)	13.7	47.3	16.6	47.8	1.21	210	14.6	30.6	39.4	145.8
Microsomal (100,000 *g*, 60 min, pellet)	5.3	18.3	6.46	18.7	1.22	123	8.5	16.1	21.0	130.5
Soluble (100,000 *g*, 60 min)	(2.1)	(7.2)	3.32	9.6	(1.58)	666	46.2	7.5	9.8	11.3
Lipid pellicle + soluble	(0.5)	(1.7)	0.96	2.8	(1.92)	370	25.7	4.9	6.3	13.1
Recovery	25.2	86.9	34.0	98.0		1795	124.6	73.5	96.8	

* Values in parentheses were detectable but too low to be measured accurately. S.A. = specific activity.

Exchange Kinetics.—Macrophages were exposed to serum medium which had cholesterol-7α-^{3}H equilibrated into the serum lipoproteins.

To eliminate variability in serum lipoproteins more than 70% of the experiments were performed using one lot number of NBCS. The pulse medium used in early experiments con-

tained 10% heat-inactivated FCS as the lipoprotein source. This serum had no lecithin-cholesterol acyltransferase activity (33), and in this concentration of serum, the cholesterol and protein content of the cells did not change over the duration of the experiment, generally 48 hr. In later experiments 20% NBCS was used since no significant difference in the pool sizes was seen and the higher serum concentration resulted in more rapid exchange.

Using 1 μCi/ml cholesterol-^{3}H (0.02 μg cholesterol/ml added to 70 μg free cholesterol/ml medium, a total of 3 ml/culture) kinetics of uptake as shown in Fig. 1 were found. Uptake of label appeared to be linear for the first 4 hr and slowly approaching an asymptote which is the equilibrium value (in this case about 8×10^4 dpm which was close to the medium specific activity of 8.5×10^4 dpm/μg of cholesterol). Since no change in cell cholesterol occurred,

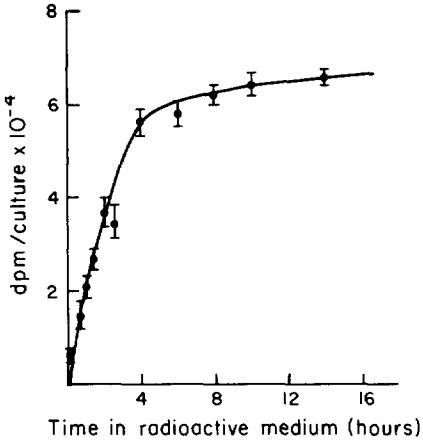

FIG. 1. Cholesterol exchange kinetics in the macrophage. Uptake of cholesterol-^{3}H into macrophages from medium containing 1 μCi/ml of cholesterol-^{3}H in 10% FCS.

label was taken up by exchange. At all times after the start of the labeling period, the label present in the cells was only free cholesterol-^{3}H as detected by TLC.

Kinetics of label washout in 10% FCS, after a 5 hr labeling in radioactive medium, are shown in Fig. 2. The loss proceeded rapidly for the first 4 hr, then more slowly. The label remaining in the cell and that appearing in the medium was free cholesterol. When the washout kinetics were plotted semilogarithmically (Fig. 3) it was clear that the curve could be fitted as the sum of at least two exponential components, a rapidly decaying component 1, and a slowly decaying component 2, which have time zero intercepts on the ordinant designated K_λ and K_μ respectively, and half-lives of $t_{1/2\lambda}$ and $t_{1/2\mu}$. If it is assumed that at the beginning of the experiment the cell is in complete isotopic equilibrium with the medium, then the biphasic washout is indicative of the presence of two subcellular exchangeable cholesterol compartments. The procedure

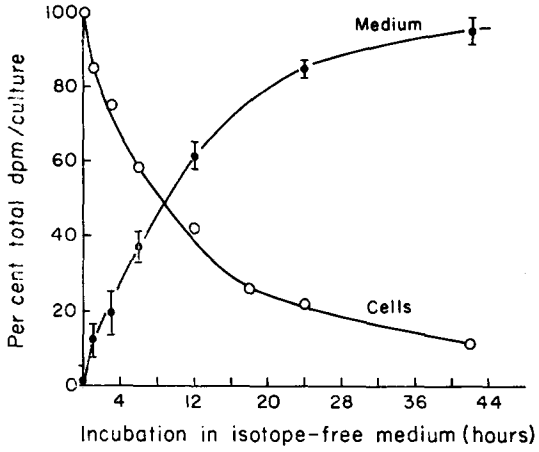

FIG. 2. Washout of cholesterol-^{3}H label from macrophages into 10% FCS medium.

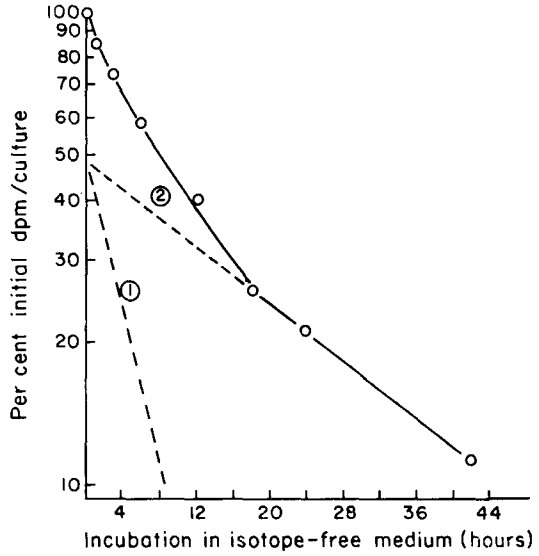

FIG. 3. Semilogarithmic plot of washout of cholesterol-^{3}H from macrophages into 10% FCS. The experimental curve (open circles) is separated into two exponential phases. The slow exponential (2) is determined by extrapolating the linear portion from 18–42 hr back to time zero. The intercept gives K_μ. The fast exponential (1) is determined by subtracting curve 2 from the experimental curve. Its intercept at time zero gives K_λ.

is described in the Appendix. The relative sizes of the two compartments can be calculated from the uptake kinetics by extrapolating to the isotopic equilibrium value (E) at infinite time, and checking this with the specific activity of the medium. The specific activity of the medium cannot be used as the equi-

librium value since physical form of the radioactive cholesterol, other than its association with lipoprotein, is unknown; in practice this value was of the same order of magnitude as E. The curve fitting used to obtain the kinetic parameters is shown in Fig. 4. The calculation of the pool sizes from uptake data does not require an equilibrium assumption.

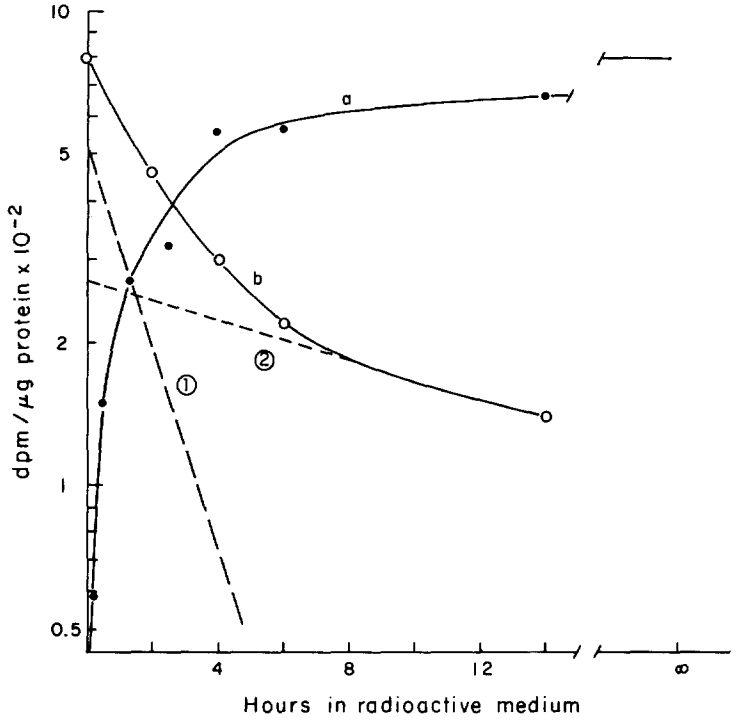

FIG. 4. Semilogarithmic plot of uptake of cholesterol-^{3}H into macrophages from labeled 10% FCS. The experimentally observed uptake curve, *a* (solid circles), is extrapolated to infinite time. The difference between the equilibrium value and the observed uptake, gives a curve, *b* (open circles), which can be divided into fast (1) and slow (2) exponential phases. The time zero intercepts of the two exponentials give K_λ and K_μ, respectively.

The kinetic parameters for both the uptake and washout experiments (Figs. 3, 4) are shown in Table II. Both methods gave the same results for the pool sizes. The half-lives of the exponential components were somewhat variable from experiment to experiment. There was considerably leeway in assigning the values of K_λ, K_μ by curve fitting. This error was difficult to evaluate since regular averaging techniques could not be applied when weighting logarithmic data.

Effect of Approximation to Isotopic Equilibrium on Kinetic Parameters and Pool Sizes.—As shown in Table II, both the uptake and washout exchange experiments gave similar results for the sizes of the exchangeable compartments. However, the chase experiments had greater reproducibility because there was no contamination of the cells by highly radioactive medium which produced errors in the uptake studies. Experiments were then carried out to measure the error produced by not having attained isotopic equilibrium at the start of the washout. Cells labeled in 20% NBCS for 2, 6, and 22 hr were then placed in

TABLE II

Kinetic Analysis of Cholesterol-^3H, Uptake, and Washout Curves

Experiment	Kinetic parameters*				Relative compartment sizes	
	K_λ	$t_{\frac{1}{2}\lambda}$	K_μ	$t_{\frac{1}{2}\mu}$	"Fast pool"	"Slow pool"
	% total	*hr*	*% total*	*hr*	*% total*	*% total*
Uptake	63.5	1.5	36.5	15	70.5	29.5
Washout	51	4.1	49	17.2	70.8	29.2

* K_λ, intercept of "fast" exponential component at time zero; K_μ, intercept of "slow exponential component at time zero; $t_{1/2\lambda}$, half-life of "fast" exponential component; $t_{12\mu}$ half-life of "slow" exponential component.

TABLE III

Effect of Labeling Time on Pool Sizes

Time of labeling in cholesterol-^3H medium	Graphical analysis				Relative compartment size	
	K_λ	$t_{\frac{1}{2}\lambda}$	K_μ	$t_{\frac{1}{2}\mu}$	Fast pool	Slow pool
hr	%	*hr*	%	*hr*	%	%
2	50	2.7	50	14.0	68.4	31.6
6	40	2.1	60	13.0	58.4	41.6
22	35	2.5	65	13.5	59.3	40.7

unlabeled 20% NBCS, and the kinetics of washout followed. Table III summarizes the results. Cells labeled for only 2 hr were farthest from isotopic equilibrium. In this case the slow pool represented 30% of the total cholesterol as compared to 40% for the cells labeled for 6 and 22 hr (slow pool size of 40% was the consistent finding for cells grown in this particular batch of serum). There was no significant change in the time constants of the two exponential components. The difference resulted from a decreased value of K_μ. Labeling times of at least 4 hr therefore produced consistent values for the relative compartment sizes.

Effect of Serum Concentration on the Kinetics of Exchange.—The effect of

serum lipoprotein concentration on exchange kinetics was next examined. When the amount of label was kept constant and the amount of serum lipoprotein in the medium was varied, thereby varying the relative specific activity of the medium, the resulting initial rate of label uptake by exchange exhibited first order kinetics. The maximum rate determined from a Lineweaver-Burke plot was about twice the rate with 20% FCS, and occurred near 100% serum (Table IV). Label added to 1% albumin medium gave inconsistent uptake results.

Macrophages prelabeled for 5 hr in 10% FCS medium were then exposed to nonradioactive medium containing either serum concentrations or 1% bovine serum albumin (BSA) in TC 199 as a control for no serum lipoprotein. Loss of label from macrophages was also serum dependent (Fig. 5). Albumin did not serve as a cholesterol-^{3}H receptor. The small loss seen may be due in part to

TABLE IV

Serum Concentration Dependency of Cholesterol-^{3}H Uptake

Serum concentration	Cholesterol-^{3}H in medium	(R.S.A.)$^{-1}$ of medium*	Initial Rate of cholesterol-^{3}H uptake	Rate corrected for R.S.A. of medium
% FCS (v/v)	*μCi/ml*		*dpm/flask per hr*	
1	1	1	3.68×10^4	3.68×10^4
10	1	10	3.00×10^4	30.0×10^4
20	1	20	2.60×10^4	52.0×10^4
30	1	30	1.48×10^4	44.4×10^4
		Maximum	1.00×10^4	100.0×10^4‡

* R.S.A. = relative specific activity = μCi/(ml × % serum).

‡ Maximum rate obtained by a Lineweaver-Burke plot of 1/(serum concentration) vs. 1/(rate) where maximum rate is derived from the intercept with the ordinant.

trace contamination of Cohn fraction V albumin with α-globulins. When the parameters derived from the graphical analysis were used to calculate the relative compartment sizes (Table V), there was no significant effect of the serum concentration used during the washout on the pools. The larger errors involved in curve fitting of the 1% FCS washout made the pool size difference not significant.

Effect of Cultivation Time on the Exchange Kinetics.—The kinetics of exchange and the sizes of the exchangeable cholesterol compartments were examined at various times of in vitro cultivation in 20% NBCS. Macrophages cultivated for 2–72 hr before exchange labeling incorporated cholesterol-^{3}H to the same intracellular specific activities. Cell size, protein content, and cholesterol content increased, although the cholesterol to protein ratios remained constant (Table VI *a*.) The relative sizes of the subcellular cholesterol compartments did not change between 24 and 72 hr of cultivation in 20% NBCS; absolute sizes double since both cholesterol and protein increased during the experiment

(Table VI *b*). The significance of differences in the time constants could not be assessed since this parameter was quite variable. However, cells cultivated in 40% serum medium had a significantly larger slowly exchanging compartment. The relative size of the slowly exchanging compartment increased further be-

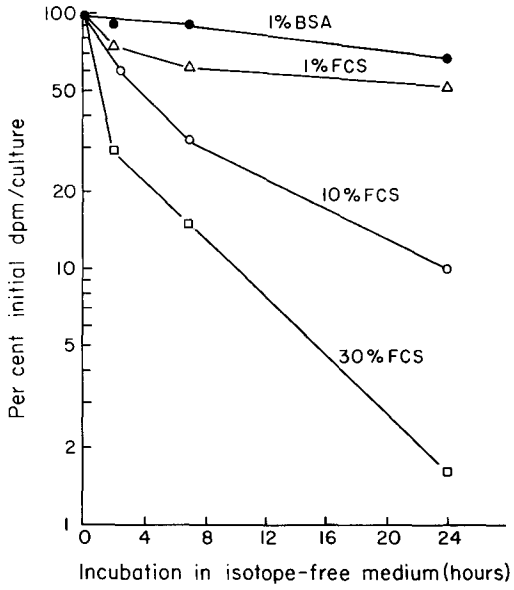

FIG. 5. Effect of serum concentration on the rate of washout of cholesterol-^{3}H from macrophages.

TABLE V

Effect of Serum Concentration on Compartment Sizes

Serum concentration in washout medium	Observed kinetic parameters				Relative compartment sizes	
	K_λ	$t_{\frac{1}{2}\lambda}$	K_μ	$t_{\frac{1}{2}\mu}$	Rapidly exchanging pool	Slowly exchanging pool
% FCS (v/v)	*%*	*hr*	*%*	*hr*	*% total*	*% total*
1	35	10.0	6.5	50	61.4	38.6
10	51	1.85	49	18.5	69.1	30.9
30	63	0.4	37	5.3	68.5	31.5

tween 24 and 72 hr of cultivation. Cells cultivated in high serum also had a higher cholesterol to protein ratio (1.4 ± 0.2 μg/100 μg protein compared to 1.2 ± 0.18 μg/100 μg protein for cells cultivated in 20% NBCS), and many secondary lysosomes (45, 46).

Effect of Inhibitors on the Exchange Kinetics.—From calculations based on the work of Ehrenreich and Cohn (32, 47) it was likely that less than $\frac{1}{10}$ of

1% of the cholesterol-^{3}H was entering the cells by nonspecific pinocytosis of serum lipoprotein. Inhibitor experiments were performed to assess the contribution of selective pinocytosis, and of metabolic functions on the uptake of labeled cholesterol. Cells in 20% NBCS were exposed to doses of inhibitor which effectively abolished the given function without injuring the cells over the duration of the experiment (47, 48) as shown in Table VII. Inhibitors of pinocytosis and protein synthesis had no effect on cholesterol incorporation during a 4 hr exposure in the presence of isotope. Puromycin and cycloheximide had significant effects after 5 hr of continuous exposure (46).

TABLE VI

Effect of Cell Age on Cholesterol Exchange Kinetics

(*a*) Uptake

Cell age	Cultivation medium	Labeling time in 10% FCS	dpm/flask $\times 10^{-4}$	dpm/100 μg protein $\times 10^{-4}$
hr	*% NBCS*	*hr*		
2	20	4	4.2	4.7
12	20	4	5.1	4.4
24	20	4	5.7	4.9
72	20	4	7.3	4.6

(*b*) Washout

Cell age	Cultivation medium	Observed kinetic parameters				Relative pool sizes	
		K_λ	$t_{\frac{1}{2}\lambda}$	K_μ	$t_{\frac{1}{2}\mu}$	Fast pool	Slow pool
hr	*% NBCS*	*%*	*hr*	*%*	*hr*	*% total*	*% total*
24	20	51	1.9	49	13.4	70	30
72	20	40	2.6	60	10.5	69	31
24	40	48	1.8	52	15.0	61	39
72	40	38	2.1	62	16.3	54	46

Both subcellular compartments were labeled in the presence of the inhibitors. This was shown by a washout experiment performed after labeling of the cells in the presence of sodium fluoride, and also by fractionation after labeling in the presence of fluoride (46).

Requirement for Live Cells.—It has been reported that significant cholesterol fluxes occurred in tissue culture cells (19) and in organ cultures of aorta (49) after killing the cells by heating. To test this possibility, cells were killed by exposure to 1.25% glutaraldehyde in phosphate-buffered saline (PBS) or by heating to 56°C for 30 min before exposure to radioactive medium. After 4 hr at 37°C, the glutaraldehyde-killed cells had taken up 17% of the label taken up by control cultures. The heat-killed cells took up 18% of the control value

Adsorption to glass in the absence of cells was less than 0.5% of the control. In both experiments, it was impossible to rule out artifactual nonspecific adsorption of labeled lipoproteins to the denatured cell surfaces.

Effect of Temperature.—The effect of temperature on the rate of uptake of labeled cholesterol was explored. Both the cells and the radioactive medium were equilibrated to required temperature before starting the experiment. The rate of uptake of label on an Arrhenius plot (Fig. 6) gave a single exponential relationship. The rate of exchange obeyed the Arrhenius equation

$$k = Ae^{-E_a/RT}$$

where k is the rate, E_a is the activation energy, T is the temperature in degrees

TABLE VII

Effect of Inhibitors on Incorporation of Cholesterol-^3H into Macrophages

Inhibitor	Concentration	Incorporation in 4 hr	Inhibition
		dpm/flask	
None		9350 ±920*	0
NaF	0.01 M	8700 ±815	N.S.‡
Dinitrophenol	10^{-5} M	8230 ±1040	N.S.
Puromycin	0.5 μg/ml	9470 ±1085	N.S.
	5 μg/ml	9100 ±425	N.S.
Cycloheximide	2 μg/ml	9270 ±220	N.S.
p-Fluorophenylalanine	400 μg/ml	9280 ±360	N.S.
Actinomycin D	0.1 μg/ml	8990 ±565	N.S.
Colchicine	50 μg/ml	8810 ±780	N.S.
Anaerobiosis	5% CO_2, 95% N_2	9010 ±890	N.S.

* Number after ± sign indicates range of values.

‡ N.S., not significant.

Kelvin, R is the gas constant, and A is the maximum rate at infinitely high temperature. For exchange, the activation energy is 12 kcal/mole. The rate doubled for every 10°K rise in temperature ($Q_{10} = 2$). At temperatures above 37°C, the rate dropped off precipitously and both inactivation of exchange and cell death occurred.

Cholesterol Synthesis in Macrophages.—Cholesterol synthesis was studied by cultivating macrophages in medium containing 2 μCi/ml acetate-1-^{14}C in the presence and absence of endogenous serum cholesterol. Endogenous acetate present in serum was removed by dialysis against saline. Total incorporation decreased in the presence of endogenous serum acetate (Table VIII) and also in 1% BSA medium which is rich in free fatty acids as an acetate source. In all cases about 15% of the ^{14}C was incorporated into chloroform:methanol (2:1) soluble lipid. When these extracts were chromatographed on thin-layer plates

few counts cochromatographed with cholesterol, and no counts with cholesterol esters. Counts were associated with phospholipids, diglycerides, and triglycerides. The maximum incorporation into free cholesterol was less than 0.01 μg cholesterol/flask in 6 hr, or less than $\frac{1}{10}$ of 1% of the cell cholesterol/hr. This influx would have no effect on the cholesterol kinetics (13).

Role of Lipoproteins in Cholesterol Exchange.—The role of various serum components in exchange was examined by electrophoresis studies with cholesterol-^{3}H-labeled serum, and by supplementing medium with isolated serum components. NBCS showed only one major band corresponding to α-lipopro-

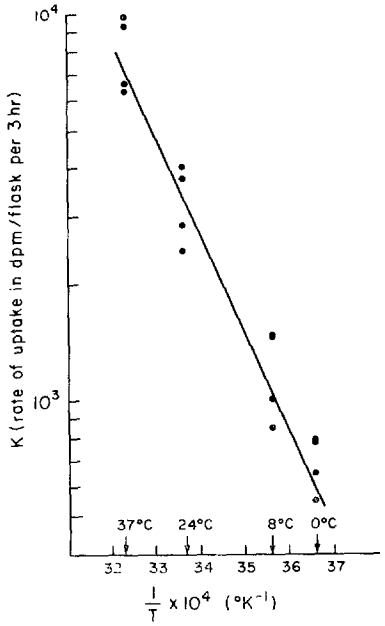

FIG. 6. Effect of temperature on the rate of uptake of cholesterol-^{3}H.

tein, although a faint β-lipoprotein band was seen occasionally (Fig. 7). Analyses indicate a paucity of β-lipoprotein in this species (50). Radioactivity measurements indicated that most of the cholesterol-^{3}H was associated with α-lipoprotein. Disc gel electrophoresis revealed a more complex banding pattern with lipoprotein bands corresponding to the major protein bands for α- and β-globulins. Most of the labeled cholesterol was associated with the α-lipoprotein band. However, a considerable proportion of the protein and label did not migrate into the gels. The major α_1-globulin band corresponded to the heavily staining Sudan black B band. Cholesterol-^{3}H recovered on serum lipoproteins during washout experiments behaved electrophoretically similarly to serum lipoproteins labeled by addition of cholesterol in acetone.

Cholesterol "acceptor" activity of serum fractions was tested. Macrophages labeled for 4 hr by exchange were placed in medium containing serum or Cohn fractions of serum for 4 hr, and the loss of label into medium was determined (Table IX). The α_1-globulin fraction was most efficient at promoting the exchange of label from the macrophage. The β-globulin fraction also had significant activity, and albumin and γ-globulins had no activity.

DISCUSSION

The cultivated mouse macrophage contains 12 μg free cholesterol/mg protein. This value is quite high as compared with other cells (21, 46), remains constant during in vitro cultivation, and is independent of the cholesterol content of the extracellular medium (18, 51, 52). The macrophage does not divide under these conditions but gradually increases in size, protein, enzyme content,

TABLE VIII

Incorporation of Acetate-^{14} into Macrophages

Experiment*	Acetate-^{14}C incorporated				
	Total‡	C-M soluble§		Cholesterol‖	
	dpm/flask $\times 10^{-3}$	*dpm/flask* $\times 10^{-3}$	%	*dpm/flask* $\times 10^{-2}$	%
1. 20% NBCS/TC 199	6.3	0.94	14.9	0.5	<1
2. 20% NBCS (dialyzed)/MEM	21.9	3.06	14.1	1.5	<1
3. 1% NBCS (dialyzed)/MEM	22.6	4.85	21.5	2.1	<1
4. 1% BSA/MEM	6.9	1.12	16.1	0.6	<1

* Medium contained 2 μCi/ml acetate-1-^{14}C (22.3 mCi/mole).

‡ Cells were incubated in radioactive medium for 6 hr.

§ C-M, chloroform-methanol, 2:1 (v/v).

‖ ^{14}C-radioactivity which cochromatographed with cholesterol standard.

and the amount of total free cholesterol. The large amounts of cholesterol may be partially explained by the presence of sterol-rich lysosomal membranes which accumulate as a result of endocytic activity. Essentially all of the cholesterol is associated with membranous elements and only trace amounts are present in a soluble form. From the studies to be reported in a companion article (46) macrophage cholesterol can be accounted for by its content in plasma membrane and lysosomal membrane. In contrast with studies on other cultured cells (21, 52, 53) no cholesterol ester could be detected by sensitive methods. This disparity may be because of the more efficient removal of serum constituents from washed cells or the presence of an active cholesterol esterase which would hydrolyze esters entering by bulk transport (54).

Under conditions of in vitro cultivation, macrophages rapidly exchange their membrane cholesterol with the cholesterol of lipoproteins contained in calf serum. In the presence of 20% NBCS about 30% of the cells' total cholesterol

is exchanged in 1 hr and more than 80% by 4 hr. By 4 hr the specific activity of cellular cholesterol had approached that of the medium and the specific activities of subcellular fractions were essentially the same. Exchange occurs in the absence of any net change in cholesterol content and proceeds in two distinguishable phases, an early rapid exchange followed by a slower component. Calculations derived from exchange kinetics were based upon a two compartment model. These indicated that the rapidly exchanging cholesterol compartment represented 60–70% of the total cell free cholesterol, and the slowly exchanging compartment accounted for 30–40%. The validity of this model depended upon the assumptions that the two compartments were linked to the extracellular medium via the rapidly exchanging compartment, that the medium reservoir of lipoprotein cholesterol was infinite so that reincorporation of tracer was insignificant, that mixing within compartments was rapid, and that kinetic analyses were performed at isotopic equilibrium. These assumptions have largely been met. As mentioned before the theoretical errors involved in the equilibrium and reincorporation assumptions were trivial, much smaller than the experimental errors derived from curve fitting and would modify the size of compartments by less than 2%. The mixing assumption is more difficult to validate but is probably reasonable in view of the rapid mixing of membrane markers in heterokaryons (55, 56).

The mechanism by which cholesterol exchanges is presumably mediated by physicochemical interactions between lipoproteins (57). Since the relationship between serum lipoprotein concentration and exchange rate exhibited first-order kinetics, it is likely that soluble lipoprotein-cell membrane interactions facilitate exchange. This suggests that cholesterol may shuttle between lipoprotein and membrane by diffusion, the rate being determined by thermal energy. This is in keeping with an activation energy of 12 kcal/mole, a value consistent with either facilitated diffusion (58) or enzyme-mediated reactions (59). It is of interest that the exchange of cholesterol between classes of lipoproteins has also been found to be temperature dependent (60).

It is quite unlikely that the bulk transport of lipoproteins, mediated by pinocytosis or phagocytosis, plays any significant role in either the uptake or

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FIG. 7. Electrophoretic separation of cholesterol-^{3}H-labeled NBCS lipoproteins. (*a*) Paper electrophoresis of two batches of NBCS. Only one major Oil Red O staining band, corresponding to α-lipoproteins, is seen. In the upper strip a faint band corresponding to β-lipoproteins can also be seen. The distribution of cholesterol-^{3}H in the serum shown in the lower strip demonstrated a single peak. (*b*) Disc gel electrophoresis of NBCS using 5% polyacrylamide. Gels 1, 2, and 3 were stained for protein with Coomassie blue after electrophoresis. The β- and α_1-globulins each have one major band plus an albumin contaminant. Serum lipoproteins run on gel 4 were prestained with Sudan Black B. There is one major lipoprotein band corresponding to the major band of α-globulins, and several smaller bands. Most of the radioactivity that traveled into the gel is found in the major lipoprotein band.

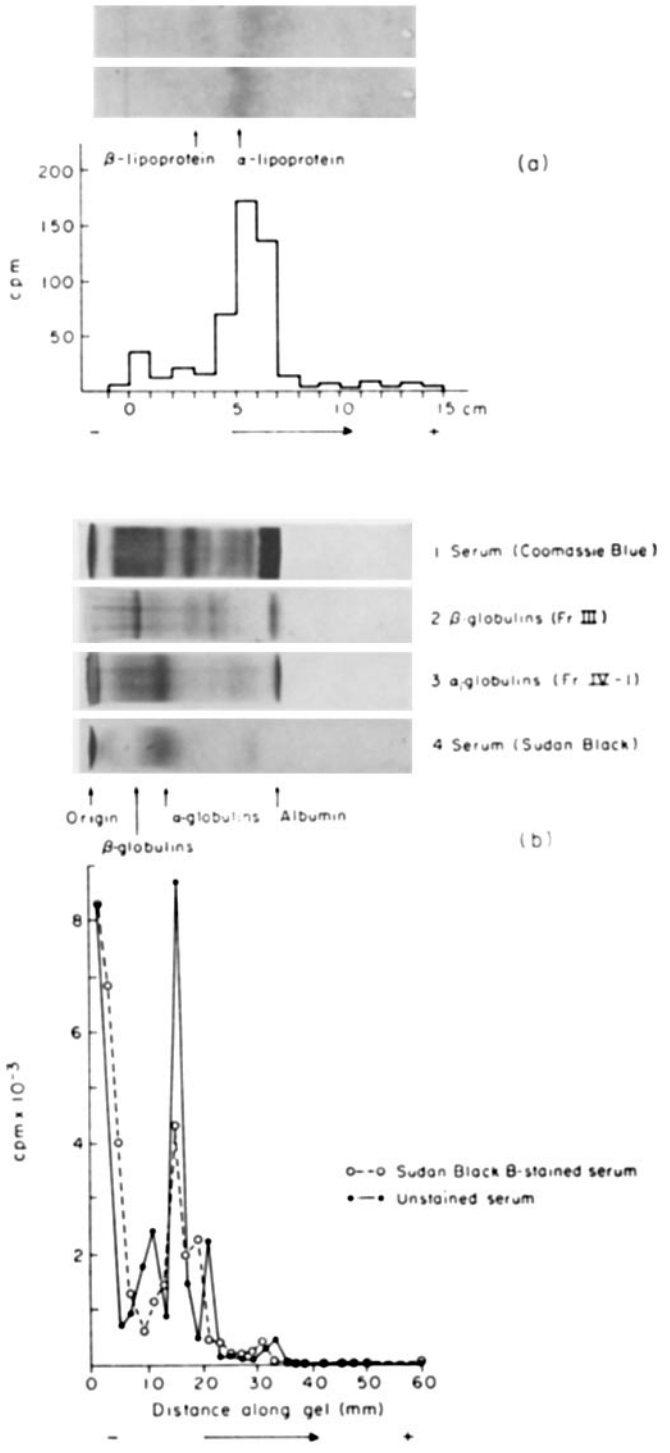
β-lipoprotein
α-lipoprotein
(a)
200
150
100
50
cpm
0
5
10
15 cm
1 Serum (Coomassie Blue)
2 β-globulins (Fr III)
3 α-globulins (Fr IV-1)
4 Serum (Sudan Black)
Origin
α-globulins
Albumin
β-globulins
(b)
8
6
4
2
cpm x 10⁻³
o--o Sudan Black B-stained serum
•—• Unstained serum
0
10
20
30
40
50
60
Distance along gel (mm)

FIG. 7

distribution of cholesterol-^{3}H by macrophages. Agents which inhibited both forms of endocytosis by more than 90% had no influence on the uptake of cholesterol-^{3}H nor did they modify the labeling of either the rapidly or slowly exchanging compartments. Similarly, exocytosis or reverse pinocytosis cannot account for the export of cholesterol. This conclusion is based upon experiments in which high molecular weight, nondegradable, intralysosomal markers are quantitatively retained within the macrophage for many days (61–63). It therefore appears that cholesterol molecules are exchanged in the absence of membrane translocations and independent of their membrane "carriers." The nature of membrane carriers for cholesterol exchange are as yet unknown.

TABLE IX

"Acceptor" Activity of Serum Fractions in Cholesterol Exchange

Medium*	Concentration	Loss of cholesterol-^{3}H	Acceptor activity‡
	mg/ml	*%/4 hr*	*units/ml per mg protein*
Albumin (Cohn fraction V), alone	5	4	0.35
α_1-globulins (fraction IV-1)	1	41	17.8
α_2-glycoprotein (fraction VI)	0.5	3	2.6
α_4-globulins (fraction IV-4)	1	13.2	5.7
β-globulins (fraction III)	1	16.5	7.2
γ-globulins (fraction II)	2	1	0.22
Combined serum fractions§	12.5	37	—
20% NBCS	—	46	20
20% FCS	—	41	—

* All media were 0.5 g/100 ml in albumin (Cohn fraction V).

‡ 1 unit = loss in 1% NBCS in 4 hr (2.3% of initial dpm).

§ Albumin, 5 mg/ml; α_1-globulins, 1 mg/ml; α_2-glycoprotein, 0.5 mg/ml; β-globulin, 1 mg/ml; γ-globulin 5 mg/ml.

They are, however, not linked to energy metabolism for short term exchange but do require a native cell surface for their expression (46).

The major protein class of calf serum which participated in cholesterol exchange were the α-lipoproteins. These proteins are the predominant lipoproteins of bovine serum and have also been implicated in the cholesterol metabolism of other cultured cells (22, 52, 64). From studies with erythrocytes, both α- and β-lipoproteins of human serum participate in exchange reactions (65). It has been suggested that because of their high phospholipid:cholesterol ratio the α-lipoproteins are relatively depleted in cholesterol and make particularly avid acceptors for cell cholesterol (22). However, because of the marked species differences in lipoproteins our observations with bovine serum cannot be extrapolated to human models.

It is difficult to relate the turnover of cholesterol in cultured cells to the

more complex conditions present in the whole organism. One obvious difference is the lack of appreciable biosynthesis of cholesterol in many primary cell cultures (66) whereas large amounts are formed in liver, skin, and intestine (14) and may contribute to membrane cholesterol. Another is the compartmental analysis of organismal cholesterol, present in a wide spectrum of cell types each with a somewhat different access to soluble lipoproteins. In the whole animal, the exchange of cholesterol from plasma to the tissues fits a model of two exchanging pools (13) with an additional nonexchanging or slowly exchanging compartment (15). Additional pools may be present which cannot be distinguished by the analysis of die-away curves and the morpho-

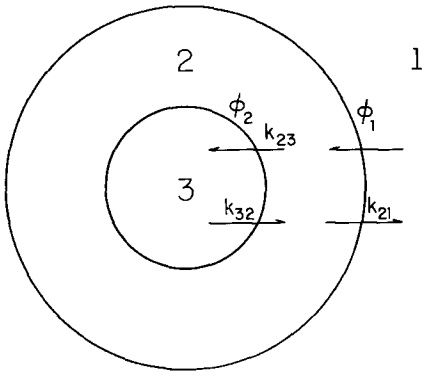

FIG. 8. Schematic drawing of the open catenary two-compartment model for cholesterol exchange in the macrophage. Compartment 1 is the reservoir which is considered infinite in size. Compartment 2 is the rapidly exchanging, or plasma membrane pool. Compartment 3 is the slowly exchanging, or intracellular membrane pool. ϕ is the flux of cholesterol (in units of mass per unit time) between the medium and plasma membrane, and is equal in both directions in the steady state. ϕ_2 is the flux of cholesterol between the plasma membrane and intracellular membranes, and is also equal in both directions. k_{21} in a constant in units of time^{-1} for flux from 2 to 1, k_{23} for flux from 2 to 3, k_{32} for flux from 3 to 2, describing the proportion of the mass of the former compartment transferred to the latter compartment in unit time.

logical identity of compartments is unknown. Under the simpler conditions of in vitro exchange, the accessibility to lipoproteins is known, and the identification of subcellular compartments can be defined. This will form the subject of the next communication in this series (46).

APPENDIX

Mathematical Model.—There are several general mathematical treatments describing the interpretation of tracer behavior in compartmentalized systems (67–69). The kinetics of an open three-compartment system used to interpret the behavior of cholesterol is taken from Huxley (70) and is described in greater detail elsewhere (71).

The data obtained for cholesterol exchange suggested a catenary three-compartment open system. This model consists of two interconnected compartments 3 and 2, with compartment 2 communicating with an infinite reservoir, compartment 1 (Fig. 8). Conditions are such that

no net mass flow occurs between the cell compartments 3 and 2 nor between compartments 2 and 1. This assumption of steady state allows us to consider the mass flux between compartments equal in both directions. The model also requires the assumption that compartment 2, which communicates with the reservoir is the rapidly exchanging compartment and compartment 3 is the slowly exchanging compartment, since two exponential components are observed. The kinetic analysis is based on the assumption that isotopic steady state has been achieved at the start of the experiment. The theoretical error generated by this assumption is less than 2% (71). The die-away curve is described by

$$c_T = K_\lambda e^{-\lambda t} + K_\mu e^{-\mu t}, \quad (1)$$

where $c_T = c_2 + c_3$, is the total cellular radioactivity (in per cent of initial radioactivity), the sum of the radioactivity in the two cellular compartments remaining at time t. K_λ and

TABLE X

*Experimentally Determined Kinetic Parameters**

Parameters	Experiment		
	1	2	3
Measured			
K_λ, *% total* (time zero intercept of rapid exponential)	54.5	60	54
K_μ, *% total* (time zero intercept of slow exponential)	45.5	40	46
$t_{1/2\lambda}$, *hr* (half-life of fast exponential)	4.1	1.6	2.0
λ, hr^{-1} (time constant of fast exponential)	0.169	0.434	0.346
$t_{1/2\mu}$, *hr* (half-life of slow exponential)	20.5	15.0	21.0
μ, *hr* (time constant of slow exponential)	0.034	0.046	0.033
Calculated			
S_2, *% total* (mass in rapidly exchanging compartment)	71.8	68.4	62.8
S_3, *% total* (mass in slowly exchanging compartment)	28.2	31.6	37.2
k_{21}, hr^{-1} (rate constant for flux from rapidly exchanging compartment to reservoir)	0.152	0.392	0.315
k_{32} hr^1 (rate constant for flux from slowly to rapidly exchanging compartments)	0.038	0.051	0.036
k_{23}, hr^{-1} (rate constant for flux from rapidly to slowly exchanging compartments)	0.028	0.037	0.027

* These parameters were determined for washout experiments using normal peritoneal macrophages prelabeled for 5 hr, then placed into 20% NBCS medium for at least 48 hr.

K_μ are the observed time zero intercepts of the two exponential components (in per cent), and λ and μ (hours^{-1}) are the time constants of the exponential components given by

$$\lambda = \frac{\ln 2}{t_{1/2\lambda}}, \qquad \mu = \frac{\ln 2}{t_{1/2\mu}},$$

where the half-lives are observable. The movement of label in each compartment is not equal to a single exponential term, but to a partial sum of the two exponential terms of equation 1. The relative size of the slowly exchanging compartment S_3 may be specified by normalizing, $K_\mu + K_\lambda = 100\%$, $S_2 + S_3 = 100\%$, thus

$$S_3 = \frac{K_\lambda K_\mu (\lambda - \mu)^2}{K_\lambda \lambda^2 + K_\mu \mu^2}, \tag{2}$$

and $S_2 + 100\% - S_3$. The relative sizes of the two compartments can also be calculated from the uptake kinetics, which can be described by

$$c_T = E - (K_\lambda e^{-\lambda t} + K_\mu e^{-\mu t}), \tag{3}$$

where E is the isotopic equilibrium value. The rate constants may be explicitly calculated using the relationship

$$k_{21} = \frac{K_\lambda \lambda^2 + K_\mu \mu^2}{K_\lambda \lambda + K_\mu \mu}, \tag{4}$$

where the rate constant describes the fraction of the mass in compartment 2 transferred to the reservoir, compartment 1, in unit time. The proportion of the total cellular cholesterol transferred in unit time is $k_{21}(S_2/[S_2 + S_3])$, since the loss of radioactivity occurs only through compartment 2. The other rate constants are functions of k_{21}.

$$k_{32} = \frac{\lambda \mu}{k_{21}} \tag{5}$$

where k_{32} is the fraction of the mass in compartment 3 transferred to compartment 2 in unit time.

$$k_{23} = \lambda + \mu - k_{32} - k_{21}, \tag{6}$$

where k_{23} is the fraction of the mass in compartment 2 transferred to compartment 3 in unit time. The use of the observed kinetic parameters to calculate pool sizes and flux rates is shown in Table X. The mass fluxes may be calculated from the relationships,

$$\phi_1 = k_{21} S_2, \quad \text{and} \quad \phi_2 = k_{23} S_2 = k_{32} S_3.$$

SUMMARY

The cholesterol metabolism of homogeneous populations of mouse peritoneal macrophages was evaluated under in vitro conditions. Macrophages are rich in free cholesterol and maintain a constant cholesterol to protein ratio (12 μg cholesterol/mg protein). No detectable cholesterol ester was present within the cell. More than 95% of total cholesterol was membrane associated and the majority was present in subcellular fractions containing lysosomes and

plasma membrane. Less than 0.1% of cell cholesterol was synthesized from acetate-1-^{14}C.

During in vitro cultivation, macrophages rapidly exchanged their membrane cholesterol with that of lipoproteins of calf serum. About 30% of the cell cholesterol was exchanged per hour in 20% serum medium, and exchange was nearly complete by 5 hr. Exchange proceeded in a rapid exponential phase followed by a slower phase. Calculations based on a two compartment model indicated that the rapidly exchanging cholesterol compartment represented 60–70% of the total cell cholesterol, and the slowly exchanging compartment accounted for 30–40%. The relationship between serum lipoprotein concentration and exchange rate exhibited first-order kinetics. The rate was determined by thermal energy, in keeping with a Q_{10} of 2, and an activation energy of 12 kcal/mole. Exchange was independent of bulk transport of lipoproteins by pinocytosis and phagocytosis, and was not linked to energy metabolism. The α-lipoproteins were the major class of proteins of calf serum participating in exchange.

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