

# EXTERNALLY DISPOSED PLASMA MEMBRANE PROTEINS

## II. Metabolic Fate of Iodinated Polypeptides of Mouse L Cells

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### ABSTRACT

The fate of the L-cell plasma membrane proteins labeled by enzymatic iodination was studied. The disappearance of label from growing cells exhibits a biphasic behavior, with 5–20% lost rapidly ( $t_{1/2} \sim 2$  h) and 80–90% lost relatively slowly ( $t_{1/2} \sim 25$ –33 h). The loss is temperature dependent and serum independent, and is accompanied by the appearance of 51% [ $^{125}$ I]monoiodotyrosine (MIT) in the medium by 47 h. A variable amount (1–14%) of acid-insoluble label can be recovered in the medium over 47 h. Sodium dodecyl sulfate (SDS)-polyacrylamide gel labeling patterns from cells cultured up to 48 h after iodination reveal no change in the *relative* distribution of radioactivity, indicating similar rates of degradation for most of the labeled membrane proteins.

The fate of the labeled membrane proteins was studied at various times after phagocytosis of nondigestible polystyrene particles. Iodinated L cells phagocytose sufficient 1.1  $\mu$ m latex beads in 60 min to interiorize 15–30% of the total cell surface area. Electron microscope autoradiography confirmed that labeled membrane is internalized during phagocytosis. The latex-containing phagocytic vacuoles are isolated by flotation in a discontinuous sucrose gradient. 15–30% of the total incorporated label and a comparable percentage of alkaline phosphodiesterase I activity (PDase, a plasma membrane enzyme marker) are recovered in the phagocytic vacuole fraction. Lysosomal enzyme activities are found in the latex vacuole fraction, indicating formation of phagolysosomes. SDS gel analyses reveal that all of the radioactive proteins initially present on the intact cell's surface are interiorized to the same relative extent. Incorporated label and PDase activity disappear much more rapidly from the phagolysosomes than from the whole cell. In the phagolysosomal compartment, >70% of the TCA-precipitable labeled proteins and all of the PDase activity are lost rapidly ( $t_{1/2} = 1$ –2 h) but  $\sim$ 30% of the labeled proteins in this compartment are degraded with a 17–20-h half-life. The slowly degraded label is due to specific long-lived polypeptides, of 85,000 and 8,000–15,000 daltons, which remain in the phagolysosomal membrane up to 40 h after phagocytosis.

Little is known about the turnover of plasma membrane proteins. Different techniques have been used (3, 4, 8, 17, 20, 25, 33) but most suffer from the necessity of isolating membranes of varying purity and yield as well as the problem of reutilization of label. Use of the enzymatic iodination technique to label only the externally disposed membrane proteins provided a reasonable solution to both problems. In the preceding report (16), we demonstrated that monoiodotyrosine (MIT),<sup>1</sup> a nonreutilisable amino acid (27), represents at least 90% of the iodinated species when intact L cells are labeled with the lactoperoxidase (LPO) system. In addition, 90% of the label has been localized to the plasma membrane after iodination, so any decrease in cell-associated radioactivity with time necessarily represents loss of label from those proteins originally present in the plasma membrane compartment.

The first part of this report deals with the fate of the incorporated radioactivity initially cell associated, and the rate of disappearance of total label and of labeled polypeptides from the L cells. The second section deals with the site of membrane protein degradation and the possible involvement of lysosomes. The enzymatic iodination of at least 15 externally disposed membrane proteins provided an excellent model system for a study of the fate of labeled plasma membrane interiorized during phagocytosis of polystyrene latex particles by L cells.

A brief report of this work has been given elsewhere (15).

## MATERIALS AND METHODS

The culture conditions, iodination, radioactivity measurements, microscopy, and autoradiography of L cells were described in the preceding report (16).

### Washout Experiments

Iodinated, rinsed L cells were resuspended in fresh 5–10% fetal calf serum-minimum essential medium (FCS-MEM) at a cell density of  $2-3 \times 10^6$ /ml, gassed with 5% CO<sub>2</sub>-95% air, and incubated at 37°C. Zero time was set at the addition of warm medium, which was actually 45 min after iodination, but cells were main-

<sup>1</sup> *Abbreviations used in this paper:*  $\beta$ NAGase,  $\beta$ -N-acetyl-glucosaminidase; BSA, bovine serum albumin; FCS, fetal calf serum; LPO, lactoperoxidase; MEM, minimum essential medium; MIT, monoiodotyrosine; PDase, alkaline phosphodiesterase I; SDS, sodium dodecyl sulfate; SP, phosphate-buffered saline; TCA, trichloroacetic acid; 2-ME, 2-mercaptoethanol.

tained at 4°C until medium was added. At various times up to 48 h duplicate 3-ml aliquots (in early experiments 5–10 ml) were withdrawn, the cells were counted and centrifuged at 1,000 rpm for 10 min at room temperature, and the medium was saved. The cells were rinsed once in 4 ml cold phosphate-buffered saline (SP) and the pellet was lysed in 0.5 ml 10% wt/vol sucrose and frozen. Duplicate 50  $\mu$ l aliquots of cells and medium from each time point were precipitated with trichloroacetic acid (TCA) onto filter disks, washed in batch, and counted. After centrifugation of the precipitable material from suitable volumes of medium or cell lysate, the TCA-soluble radioactivity was quantitated by Sephadex chromatography (14, 18).

### Phagocytosis Experiments

**PHAGOCYTOSIS OF LATEX:** L cells grown in suspension were rinsed at least twice in SP after iodination and resuspended at  $5-8 \times 10^6$  cells/ml in 5% FCS-MEM. 1.1  $\mu$ m diameter polystyrene latex particles (Dow Chemical Co., Indianapolis, Ind.) which had been washed twice in 10 vol MEM and dispersed were added to the cells at a final concentration of 1.4–2.0 mg/ml or 2,000–5,000 beads/cell. The beads and cells were incubated 30–60 min at 37°C in a spinner flask, after which they were chilled to 4°C and the cells centrifuged away from the bulk of the unphagocytosed latex particles. The pellet was resuspended in cold MEM or SP and the cells were centrifuged at 300 g (1,200 rpm) for 15 min through a 20–30 ml cushion of 4% bovine serum albumin (BSA) or 50% FCS which retarded uningested particles. The cells were then rinsed twice in SP.

The number of beads ingested per cell was quantitated on thick sections (0.4  $\mu$ m) of fixed, Epon-embedded cells by counting beads in every cell in a 40-times magnified field. Over 500 beads were counted. An average L-cell diameter of 13  $\mu$ m was calculated from measurements on numerous cells photographed at low magnification in the electron microscope. A correction factor of 10 was used to obtain the total number of particles per cell from the number of particles per cell section.<sup>2</sup>

**ISOLATION OF LATEX-FILLED PHAGOLYSOSOMES:** Latex-filled cells were suspended in 5 ml 0.01 M Tris-HCl, pH 7.4 or 8.0, for 3 min on ice, homogenized with 10 vigorous strokes in a tight glass Dounce homogenizer, and MgCl<sub>2</sub> and sucrose added to final concentrations of 1.5 mM and 30% (wt/vol) respectively. The cell suspension was homogenized again till more than 90% of the nuclei were clean of latex particles and few large

<sup>2</sup> A cell of diameter 13  $\mu$ m (the average diameter of the L cell) will be found in 30 random, 0.4  $\mu$ m sections, and a latex particle of 1.1  $\mu$ m in three 0.4  $\mu$ m sections. The average number of latex particles per cell can be calculated from the average number of particles in one 0.4  $\mu$ m section  $\times \frac{30 \text{ sections/cell}}{3 \text{ sections/latex particle}}$ .

membrane sheets were visible under the phase microscope. The following steps were modified from the method of Wetzel and Korn (37) for flotation of latex-filled vacuoles. 2.7 ml of the homogenate were placed in the bottom of each of three centrifuge tubes (Beckman SW 41) and overlaid with 20% (6 ml) and 10% (3 ml) sucrose (wt/vol). After centrifugation at 110,000 *g* (30,000 rpm) for 90 min, the bulk (>95%) of the latex vacuoles was at the 10%/20% interface and the rest was in the 20%/30% interface. The 10% and 20% fractions contained little visible material when viewed with phase optics. The 30% fraction contained vesicular material and the pellet contained nuclei, a few unbroken cells, and other residue. The fractions were analyzed for protein, TCA-insoluble radioactivity, latex, alkaline phosphodiesterase I, and acid hydrolase activities. The particles of the 10%/20% interface fraction were concentrated by centrifugation at 5,400 *g* (6,000 rpm) for 20 min (Lourdes, SCR) or 7,700 *g* for 20 min (8,000 rpm, SW 41, L2) and the proteins solubilized for electrophoresis on SDS-disc polyacrylamide slab gels. The presence of latex did not interfere with the migration of proteins as assessed by Coomassie blue patterns and autoradiograms of homogenates with and without latex.

#### *Phagocytosis-Washout Experiments*

Iodinated L cells were exposed to latex for 1 h, rinsed, and resuspended in fresh 5% FCS-MEM. Zero time was set at the addition of the medium. Phagolysosomes were isolated by flotation from approximately equal volumes of cells (~150 ml) over a 20- to 44-h period and analyzed. In addition, duplicate 3 ml samples of cells were removed from the same flask and a typical washout experiment was performed. The percent of total acid-insoluble <sup>125</sup>I found in the 10%/20% interface at each time was corrected for the loss of total radioactivity from the cell lysate sample and expressed as percent of initial acid-insoluble <sup>125</sup>I. PDase activity was also measured in the total cell and isolated phagolysosomes at each time and the percentage of total initial PDase activity remaining in the 10%/20% interface was plotted.

#### *Enzyme Assays*

Alkaline phosphodiesterase I and aryl sulfatase were assayed as described (16).  $\beta$ -*N*-Acetylglucosaminidase was assayed with 4.4 mM *p*-nitrophenyl- $\beta$ -*N*-acetylglucosamine (Sigma) at pH 5.0. The liberation of *p*-nitrophenol was measured as with alkaline phosphodiesterase I. Enzyme kinetics were linear for over 2 h in the range of 0.1–10 × 10<sup>6</sup> cells. To measure total activity, 0.1 M Na acetate buffer and 0.1% Triton X-100 were included.

#### *Analytical Procedures*

Polystyrene latex was assayed by a modification of the procedure of Weisman and Korn (34). 1 vol (0.05 ml) of the sample containing latex was mixed with 19 vol (0.95

ml) of dioxane, AR or spectro quality, and incubated 30 min at 37°C. Particulate material was sedimented at 1,000 *g* for 20 min, the supernate filtered through Whatman GF/C glass fiber disks, and the absorption of the filtrate measured at 259 nm. Standards were prepared from the same latex stock and an equivalent amount of cellular protein was added in an attempt to correct for protein absorption in the sample. Other procedures were as described (16).

## RESULTS

### *Fate of Labeled Proteins*

#### CONTROLS

It was necessary to establish that iodinated cells remained viable over the course of the washout experiments and exhibited properties similar to those of uniodinated controls. The change in population density from 0 to 44 h after iodination of cells was plotted (Fig. 1). Cells (A) were iodinated as usual with <sup>125</sup>I and LPO, rinsed, and replaced in fresh 5% FCS-MEM. A second aliquot (B) was iodinated with <sup>127</sup>I (nonradioactive) and handled in parallel with the <sup>125</sup>I cells. The third aliquot (C) was neither iodinated nor rinsed but resuspended in fresh 5% FCS-MEM at the same time as A and B. The doubling times were 16, 20, and 20 h for A, B, and C, respectively, all within the 16–20-h generation time of the L cell line used. Some variability in cell number was evident in the first 4 h but was seen in both <sup>125</sup>I and control cells which had been rinsed in parallel. More than 95% of the cells from each treatment (Fig. 1) could be plated onto plastic petri dishes at each time point. In an additional experiment, cells were prelabeled with [<sup>3</sup>H]thymidine, then rinsed and iodinated with <sup>127</sup>I, rinsed but not iodinated, or neither rinsed nor iodinated. Aliquots of cells from the three treatments were analyzed for acid-insoluble [<sup>3</sup>H]thymidine over 24 h. The curves were identical for each treatment, indicating that the iodination and rinsing procedures had no adverse effect on normal cell viability.

#### DISTRIBUTION OF RADIOACTIVITY

The distribution of <sup>125</sup>I in the cells and medium over a 47-h washout experiment was examined (Table I). Constant volume (5 ml) aliquots of cells were removed at the times indicated, rinsed, and the fractions analyzed. The acid-insoluble radioactivity associated with the cells and medium at zero time was defined as 100%. 94–100% of this initially acid-insoluble radioactivity could be re-

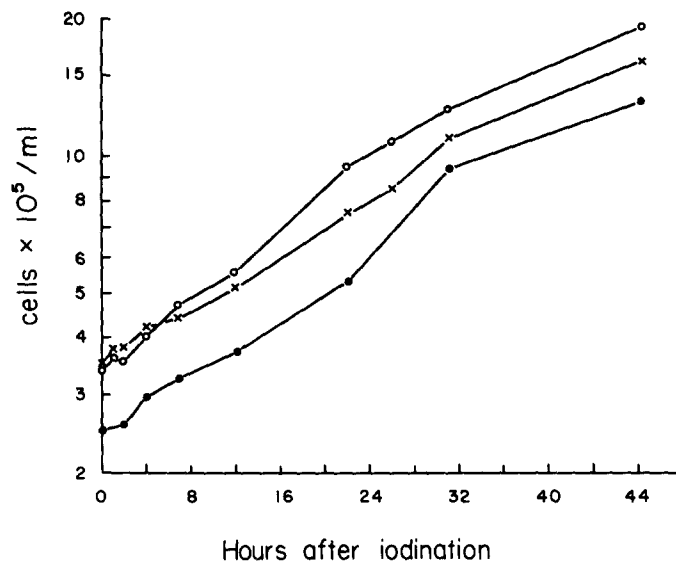


FIGURE 1 Increase in population density of L cells after iodination. A, Cells iodinated with  $^{125}\text{I}$  (O—O), B, cells iodinated with  $^{127}\text{I}$  (●—●), C, Cells untreated and resuspended in fresh FCS-MEM (x—x).

TABLE I  
Distribution of Radioactivity after Iodination

Hours after Iodination	Acid-insoluble radioactivity		[ $^{125}\text{I}$ ]Monoiodotyrosine in medium*	Recovery of 0-h acid-insoluble radioactivity
	Cells	Medium		
	%	%	%	%
0	97.4	2.6	0.5	100‡
3	82.4	5.8	8.1	96.3
20	46.5	13.0	40.5	100
47	26.4	16.5	51.0	93.9

\* Percent of zero time, acid-insoluble radioactivity.

‡ Number of acid-insoluble counts at zero time set at 100%.

covered at each time point as either cell-associated and acid-insoluble, medium-associated and insoluble, or as soluble monoiodotyrosine in the medium. The appearance of MIT indicated that degradation was the predominant fate of the labeled membrane proteins (more precisely, the labeled peptide regions of the labeled membrane proteins). In addition, quantitative recovery of the soluble label as MIT eliminated the possibility of deiodination of the labeled proteins. Some acid-insoluble label did appear in the medium, accounting for 13% of the initial radioactivity at 20 h and 16% at 47 h. The medium values in this experiment

represented an upper limit of the acid-insoluble label found in the medium in 15 washout experiments (see below).

The question of whether degradation was a cell-mediated phenomenon or due to serum proteases was analyzed by resuspending iodinated cells in either 10% FCS or 0.6% bovine serum albumin (BSA-MEM) and measuring the distribution of radioactivity over 48 h. Cell viability and generation time were not altered by incubation of cells in BSA-MEM over 48 h. The results were identical to those shown in Table I. Whether  $^{125}\text{I}$ -labeled L cells were incubated in BSA or FCS, MIT was the major iodinated species by 48 h and was found primarily in the medium. It was concluded that the degradation of labeled L-cell membrane proteins was cell mediated.

### Kinetics of Label Loss

#### ACID-INSOLUBLE RADIOACTIVITY ASSOCIATED WITH CELLS

The kinetics of disappearance of acid-insoluble label from iodinated L cells was next determined. To exclude the necessity of correcting for label dilution due to cell division, a constant volume of cells in FCS-MEM was withdrawn at each time point over the entire experiment. The cell number in a given volume increased, as indicated in Fig. 1, so the amount of label per cell decreased. How-

ever, the entire population in a given volume maintained the same *total* radioactivity, degradation or secretion being responsible for the loss of acid-insoluble label from the population. Fig. 2 represents results from 1 experiment of 15 performed. The loss of acid-insoluble  $^{125}\text{I}$  from the cells over 48 h appeared to be the sum of at least two exponential curves. Using simple graphical analysis of the two components, it was found that the fast element had a half-life of 3 h, comprising 20% of the total label present at 0 h, and the slow element exhibited a half-life of 33 h and comprised 80% of the total. Analysis of 15 similar experiments conducted over a 1-yr period revealed the size of the two components at zero time to be 5–20% for the fast and 80–95% for the slow. The half-lives ranged from 0.75 to 3 h for the fast component, with an average of 2 h and 22–35 h for

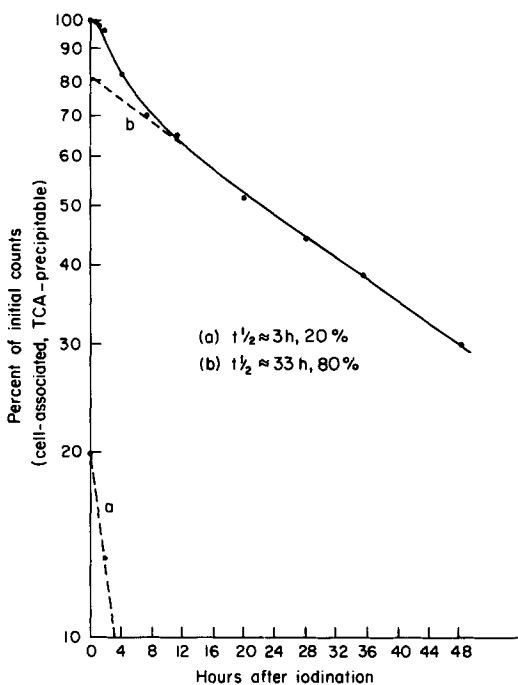


FIGURE 2 Kinetics of label loss from iodinated L cells. Semilogarithmic plot represents TCA-precipitable radioactivity remaining cell associated from 0 to 47 h after iodination. The cpm at 0 h was defined as 100%. The experimental curve (●—●) is separated into two exponential phases. The slow component (b) is determined by extrapolating the linear portion from 12 to 47 h back to time zero. The intercept gives the size of the slow component, 80% in this experiment. The fast exponential (a) is obtained by subtracting (b) from the experimental curve. Its intercept at time zero gives the size of the fast component, 20% in this experiment.

the slow. This analysis suggested that there was a rather large (80–95%) stable component to the label initially incorporated into the plasma membranes of L cells.

#### ATTEMPTS TO ALTER THE KINETICS OF LABEL LOSS

**TEMPERATURE:** As seen in Fig. 2 there was sometimes a lag of 30–90 min before significant amounts of incorporated label were lost from the cells. Since the cells had been held at 0°–4°C during rinsing and were subsequently warmed to 37°C, the temperature dependence of the loss of label was investigated. Iodinated L cells were resuspended in fresh 10% FCS-MEM at 4°C, maintained at 4°C for 4 h, then warmed to 37°C for the duration of the washout. The loss of label was compared to that from iodinated cells cultured at 37°C from 0 h (Fig. 3). No loss of label occurred when cells were kept at 4°C. After the transition to 37°C there was a 30-min lag, after which the loss was initially faster (fast component  $t_{1/2}$  was 1.5 h vs. 2 h for the control) and then paralleled the control

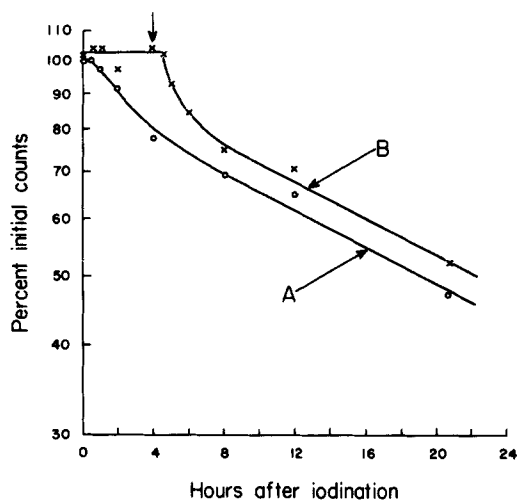


FIGURE 3 The effect of temperature on kinetics of label loss from iodinated L cells. Semilogarithmic plot represents TCA-insoluble radioactivity remaining cell associated from 0 to 24 h after iodination. A, iodinated cells resuspended in 37°C, 5% FCS-MEM at 0 h. B, iodinated cells resuspended in 4°C, 5% FCS-MEM from 0 to 4 h, then warmed to 37°C (vertical arrow) for the duration of the experiment. The counts at 0 h in each population were defined as 100%. The experimental curves were separated into two exponential phases whose sizes and half-lives were determined as described in Fig. 2. Fast component  $t_{1/2} = 2$  h, 10%; slow component  $t_{1/2} = 23$  h, 90%.

curve. The cellular events responsible for the loss of label from L cells were clearly temperature-dependent processes.

**POPULATION DENSITY, CELL DIVISION:** The influence of population density and rates of cell division were next investigated. Iodinated cells were maintained in 10% FCS-MEM until label loss was occurring at the slow rate (13 h), at which time aliquots were concentrated (to  $4.6 \times 10^8$  cells/ml), diluted (to  $2 \times 10^8$  cells/ml), or left unchanged ( $6.2 \times 10^8$  cells/ml). There was little difference in the rates of label loss from the three populations over a 24-h period although growth varied from +35% (high density cells) to +200% (low density cells, after a 6-h lag). These results indicated that neither cell division nor population density significantly altered the rate of disappearance of acid-insoluble  $^{125}\text{I}$  from L cells.

**OTHER VARIABLES:** Attempts to reduce the fast component of the washout curve were generally unsuccessful. Rinsing cells in BSA-MEM rather than SP had no effect on the label loss. Conditioned FCS medium did not alter the rate. Increasing the incorporation of iodide atoms per cell from 1,000 to 60,000 did not increase the fast component, as would have been expected if some proteins were damaged by excessive iodination and degraded more quickly than others.

#### *Acid-Insoluble Radioactivity in the Medium*

A small percentage of label which was initially cell associated could be found in the growth medium over a 48-h washout experiment. Analysis of this acid-insoluble radioactivity from five experiments revealed that a maximum 5.1–16.0% was found after 40 h of incubation and in seven experiments no label above background could be precipitated from the medium. In those experiments where label was found in the medium, most was released in the first 8 h. This release may in part explain the occurrence of a fast cellular decay component, but not completely, because in the seven experiments where no label was found, there was still a fast component. The medium from cells maintained at 4°C for 4 h contained no insoluble label above background. It was impossible to electrophorese the serum-containing medium on SDS gels due to the high concentration of albumin present and the low radioactive content of a suitable aliquot. Sephadex G-200 chromatography in 0.1% SDS and 0.1 M Na phosphate of medium from iodinated cells (2 h after addition of FCS-

MEM) indicated that most of the labeled material was eluted after albumin. The nature of the medium radioactivity was not investigated further.

#### *Gel Electrophoresis*

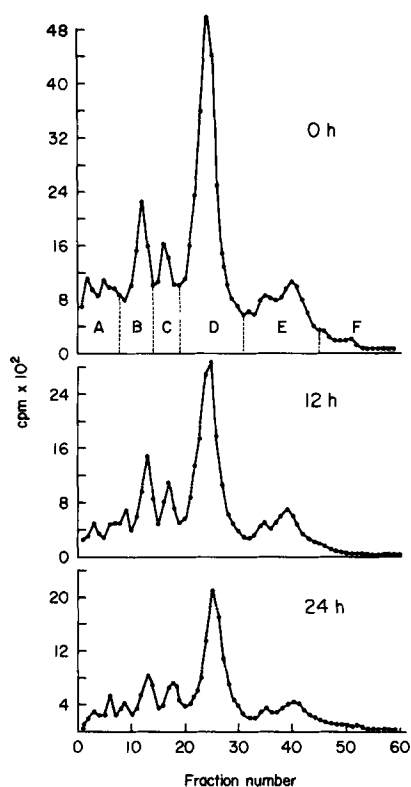
##### POLYPEPTIDES ASSOCIATED WITH L CELLS

The possibility that the fast and slow rate components represented differential degradation of labeled proteins was investigated using SDS-polyacrylamide gel electrophoresis. Aliquots of lysates from cells incubated 0–48 h after iodination were solubilized in SDS-2ME and electrophoresed in 10 cm 7.5% tube gels. The radioactivity distribution from Coomassie blue-stained gels was plotted (Fig. 4). The general labeling pattern remained unchanged from 0 to 24 h after iodination, but the total amount of radioactivity found in each of the five peaks decreased. This occurred more rapidly from 0 to 12 h than from 12 to 24 h. 30–48-h gels (not shown) yielded the same relative labeling pattern as earlier time points.<sup>3</sup> The gel radioactivity pattern was divided into the regions indicated (Table, Fig. 4) and the relative amount of label found in each calculated. Duplicate 0-h gels were electrophoresed and percentages calculated to assess the reproducibility of the method, which was accurate to 5% between experiments. These results indicated that the rates of degradation of the iodinated membrane polypeptides were very similar.

#### *Fate of Labeled Proteins after Phagocytosis*

It has been shown that >50% of the L-cell membrane proteins that could be iodinated were degraded to the level of amino acids over a period of 48 h in culture. These labeled proteins were apparently degraded at similar rates. However, if only a small percentage of the membrane was being degraded at any one time, only *very large* rate differences could be detected when total cells were analyzed. In addition, EM autoradiography of iodinated cells had revealed a variable percent (5–15%) of the total grains localized to the centrosphere region (pinocytic vesicles?) immediately

<sup>3</sup> It was difficult to obtain accurate gel labeling patterns from lysates of cells incubated more than 30 h because the population had increased ~150% while the cell-associated radioactivity had decreased to ~40% of that at 0 h. This resulted in an 85% reduction in radioactivity per unit of cell protein applied to the gel.



Hours after iodination	Region <sup>1</sup>					
	A	B	C	D	E	F
0	12.2	13.6	7.4	34.7	17.0	7.2
0 (duplicate)	13.4	18.4	8.3	38.0	16.8	8.3
3	15.7	13.5	8.1	37.6	18.8	8.7
12	14.9	12.5	9.0	37.4	18.5	9.6
24	14.0	12.1	9.5	36.0	17.2	10.5

FIGURE 4 SDS-polyacrylamide tube gels of iodinated cells. Aliquots of cells removed 0, 12, and 24 h after iodination, rinsed, the cell pellet solubilized in SDS-2ME. Gel composition 7.5% acrylamide, 35:1 A/MBA. Electrophoresis for 15 h at 6 mA/gel, gels stained for protein, cut, and 1.8-mm sections counted. Graphs represent distribution of  $^{125}\text{I}$  in the gel sections. The table presents the sum of radioactivity in each of the indicated regions (top gel) of gels from 0, 12, and 24 h after iodination, expressed as percent of total recovered label.

after iodination (16). It seemed possible that this intracellular radioactivity represented the small amount of acid-insoluble label that was degraded rapidly, that the 90% of label at the sur-

face represented the slow component, and that interiorization of label from the cell surface to the centrosphere region was the rate-determining step in degradation. Therefore, the next series of experiments was designed to compare the fate of labeled membrane which had been forcibly internalized with that of membrane remaining at the cell surface.

#### CONDITIONS AND EXTENT OF PHAGOCYTOSIS BY L CELLS

L cells were found to phagocytose  $1.1 \mu\text{m}$  latex particles under restricted conditions. Cells adherent to a surface did *not* phagocytose significant amounts of latex in 4 h, but cells in suspension would ingest large numbers in 30–60 min if high concentrations of latex (1–2 mg/ml) were used and the latex was prerinse free of adsorbed proteins. Fig. 5 *a* shows that more than 95% of the cells in a population had ingested latex in 30 min. 50–100 particles were located in the centrosphere region and around the nucleus, suggesting interiorization. Electron microscopy confirmed this localization (Fig. 5 *b*). After a 60-min pulse of latex, 80% of the cell-associated particles were within the cytoplasm as single particles surrounded by a membrane. Calculations based on the morphological quantitation generally yielded 85–150 beads ingested per cell over 1 h. The spectrophotometric assay (34) could not be employed due to the presence of large amounts of cellular material which resulted in artifactually high readings.

#### AUTORADIOGRAPHY

The relative distribution of label present in iodinated L cells before and after phagocytosis was assessed using autoradiography. L cells were iodinated as described, exposed to latex for 30 min, rinsed, fixed, and processed. The distribution of grains over cell compartments was calculated both before and after phagocytosis (Table II). An average of 200 particles had been ingested per cell, or ~47% of the cell's surface area, which correlated with the reduction in grains at the cell periphery. Most of the grains lost from the cell surface were localized to the periphery of latex vacuoles (Fig. 5 *b*). This suggested that iodinated membrane was interiorized to the same relative extent as the total plasma membrane. The average number of grains per cell decreased by 10% during phagocytosis. This reduction could be ascribed either to loss of label or to slight differences in section thickness. Control experiments indicated

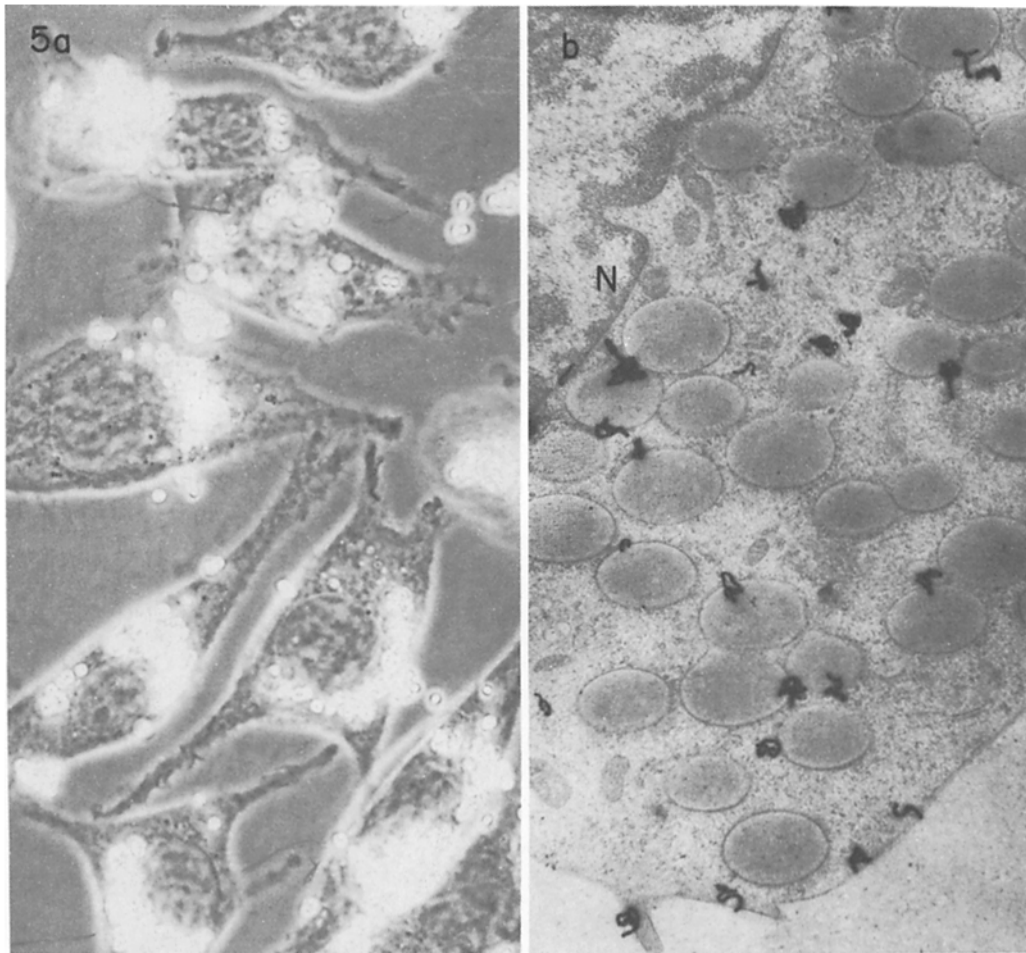


FIGURE 5a Phase-contrast micrograph of latex-filled L cells ( $\times 850$ ). Cells were exposed to 1.5 mg/ml latex for 60 min, rinsed, then allowed to settle onto a plastic surface for 2 h. Many particles are present in the centrosphere region of every cell in the field, but it is difficult to assess the number of particles actually inside the cell. 5b EM autoradiograph of iodinated L cell after ingestion of latex ( $\times 12,800$ ). Grains representing incorporated radioactivity are localized to the periphery of latex vacuoles inside the cells as well as to the cell surface. Exposure of 12 days, emulsion not removed before staining. *N*, nucleus.

that free  $^{125}\text{I}$  in the medium was not adsorbed to the latex which was ingested.

#### Isolation of Phagolysosomes

In order to identify those labeled membrane proteins which were interiorized during phagocytosis and to establish their rates of degradation, phagolysosomes were isolated from iodinated, latex-filled L cells.

#### FLOTATION THROUGH A DISCONTINUOUS SUCROSE GRADIENT

Cells were iodinated, exposed to latex as described, rinsed, homogenized, and the various

fractions obtained were analyzed for acid-insoluble  $^{125}\text{I}$ , protein, PDase, and two lysosomal enzymes (Table III, experiment A). The vast majority of latex was found in the 10%/20% interface fraction and the remainder in the 20%/30% interface fraction. PDase activity and acid-insoluble  $^{125}\text{I}$  were concentrated in the 10%/20% interface. Immediately after ingestion (0 h), 11% of both lysosomal enzyme activities were present in the 10%/20% interface fraction. This indicated some fusion between phagosomes and pre-existing lysosomes.

To assess the extent of plasma membrane contamination of the 10%/20% interface fraction, cells were preloaded with latex, then iodinated, and the



TABLE II  
Distribution of Radioactivity Before and After Phagocytosis\*

Cells	Latex Per Cell	Average Grains Per Cell Section†	Distribution			
			Periphery	Centrosphere		Nucleus
				Over Latex	Other	
Before phagocytosis	—	70	85	—	10	3
After phagocytosis	200§	60	48.5	45	4	2

\* Cells iodinated at 22°C for 20 min with the standard reagent concentrations but Na<sup>125</sup>I at 1,000 µCi/ml. After rinsing, 0.5 of the cells were fixed and processed while the other half were exposed to 2 mg/ml 1.1 µm latex particles for 30 min, then rinsed and fixed.

† Thin, gold sections coated and exposed for 12 days.

§ Interiorization as single particles with membrane surrounding each. Estimate of cell surface area interiorized around the latex beads, 47%.

TABLE III  
Analysis of Fractions from Phagolysosomal Isolation

Experiment	Fractions	Protein	Acid-Insoluble	PDase	β-NAGase	Aryl sulfatase	Latex
			<sup>125</sup> I				
		%	%	%	%		
A*	10%	0	0	0	0	0	0
	10%/20% interface	5	28.9	30.6	11.5	11.7	++++
	20%	0	3	0.1	0.1	0.1	0
	20%/30% interface	—	13	12	10	11	±
	30%	55	21	34	58	68	0
	Pellet	39	34	18	25	15	±
Recovery		99	99	94	104	106	—
B‡ Control	10%	0	0	—	0	—	0
	10%/20% interface	3.3	2.5	—	15.5	—	++++
	20%	0	0	—	0	—	0
	20%/30% interface	—	1.6	—	4.0	—	±
	30%	42	9.1	—	34.2	—	0
	Pellet	41	71	—	48.0	—	±
Recovery		85	85	ND	§	ND	—

\* Experiment A: Iodinated L cells exposed to 2 mg/ml latex for 60 min, rinsed, and latex vacuoles isolated. These cells had ingested an average of 170 beads per cell corresponding to interiorization of ~ 40% of the cell surface area.

‡ Experiment B: L cells first exposed to latex, rinsed, incubated in 5% FCS-MEM for 18 h then iodinated and latex vacuoles isolated from the cells.

§ Homogenate activity not measured, percentages are of recovered activities.

ND = not determined.

phagolysosomes immediately isolated (Table III, experiment B). Only 2.5% of the total radioactivity was found in this fraction, indicating little plasma membrane contamination.

In six experiments where phagolysosomes were isolated from iodinated L cells immediately after

the exposure to latex, 10–30% of the total cell label and similar amounts of PDase activity were found in the 10%/20% interface. The amount of label was ~80% of the amount predicted on the basis of particle counts. Latex vacuoles were never isolated free of acid hydrolase activity, even after a short

phagocytic pulse (30 min). This indicated that the membrane surrounding the latex particles was a mixture of plasma and lysosomal membrane.

### Kinetic Studies

The iodinated cells that had ingested latex and the incubation medium were both analyzed for MIT and acid-insoluble  $^{125}\text{I}$  over a 47-h experiment. Forced internalization of membrane did not alter the basic pattern of degradation of labeled membrane proteins to amino acids found in non-phagocytosing, iodinated L cells (Table I).

#### LOSS OF ACID-INSOLUBLE RADIOACTIVITY FROM CELLS AND PHAGOLYSOSOMES AFTER PHAGOCYTOSIS

Fig. 6 A presents an experiment in which phagolysosomes were isolated at 0, 3.75, 9, and 19 h after phagocytosis and the loss of label from this compartment was compared to that from the total cell.<sup>4</sup> The loss of label from the total cell displayed a characteristic biphasic curve with fast and slow decay components of 23% and 77%, respectively (Table IV, A). ~30% of the total label was associated with the phagolysosomes at 0 h and the loss of label from this compartment was much faster than from the total cell, but also biphasic. The size of the fast component was 73% and the slow, 27%. By 19 h only 3.8% of the initial label remained in the phagolysosome, a loss of 88%. In contrast, the total cell label was reduced by 60% over 19 h, and 27% of this loss was due to degradation of label found initially in the phagolysosomal compartment. 30.6% of the PDase activity was present in the 10%/20% interface fraction at 0 h and the activity decreased as a single exponential having at  $t_{1/2}$  of ~4 h (Fig. 6 A). The total enzyme activity per cell decreased by 22% between 0 and 9 h. All of this decrease could be ascribed to the loss of activity in the phagolysosomes.

A longer washout experiment was performed on L929 cells with similar results (Fig. 6 B and Table IV, B). Only 15.4% of the total label was found in the 10%/20% interface fraction after 80 min of phagocytosis, reflecting a smaller number of latex

<sup>4</sup> In these phagocytosis experiments (Fig. 6 and Table IV), a decrease in total cell-associated radioactivity reflects loss from labeled plasma membrane in the phagolysosomal compartment *plus* loss from the remainder of the plasma membrane, whether it is at the cell surface or elsewhere in the cell.

particles ingested per cell. The fast component of the total cell label loss was only 6% of the initial label, possibly reflecting the less extensive internalization of iodinated membrane by zero time. The curve representing loss of acid-insoluble  $^{125}\text{I}$  from the phagolysosomes was again biphasic with a large, fast component (61%).

The 10%/20% interface, 20%/30% interface, 30% and pellet fractions from each time point in the two phagocytosis experiments were analyzed for protein, radioactivity, and enzyme activities. The results for the 10%/20% interface, the phagolysosomal fraction, are presented in Table V. The percent of recovered acid-insoluble  $^{125}\text{I}$  decreased most rapidly in the first 2.5-4 h and then more slowly. Since only 40% of the *total initial* label remained cell associated by 19 h (Table V, experiment A), 3.8% of the initial label was still present in the phagolysosomes (Fig. 6 A). Analysis of two lysosomal enzymes,  $\beta$ -*N*-acetyl-glucosaminidase and aryl sulfatase, revealed parallel distributions over the 19-h experiment. 12% of each activity was present at time zero and the percentages increased to maxima of 41% and 33% by 3.75 h. The total activities of these two enzymes increased during the 19-h experiment (not shown), but the percentage of each found in the phagolysosomes decreased. Similar results were obtained in the 44-h phagocytosis experiment.

Incorporated label and PDase activity present in plasma membrane which was internalized into the lysosomal compartment during phagocytosis were lost (degraded) more rapidly than the total cell label.<sup>4</sup> The simplest hypothesis would involve inactivation and degradation of the labeled, externally disposed peptide segments of the membrane proteins by lysosomal enzymes. A small percentage of the membrane label persisted in the phagolysosomes for as long as 44 h and could represent continuing endocytosis of labeled membrane or the presence of labeled components with longer half-lives.

#### GEL ELECTROPHORESIS OF CELLS AND PHAGOLYSOSOMES

To investigate the possible presence of long-lived labeled components in the phagolysosomes, aliquots of iodinated cells and 10%/20% interface fractions from four time points over the 19-h phagocytosis experiment were electrophoresed. The gels were stained for protein, dehydrated, and autoradiographed (Fig. 7). The Coomassie blue pattern of the homogenate increased in intensity

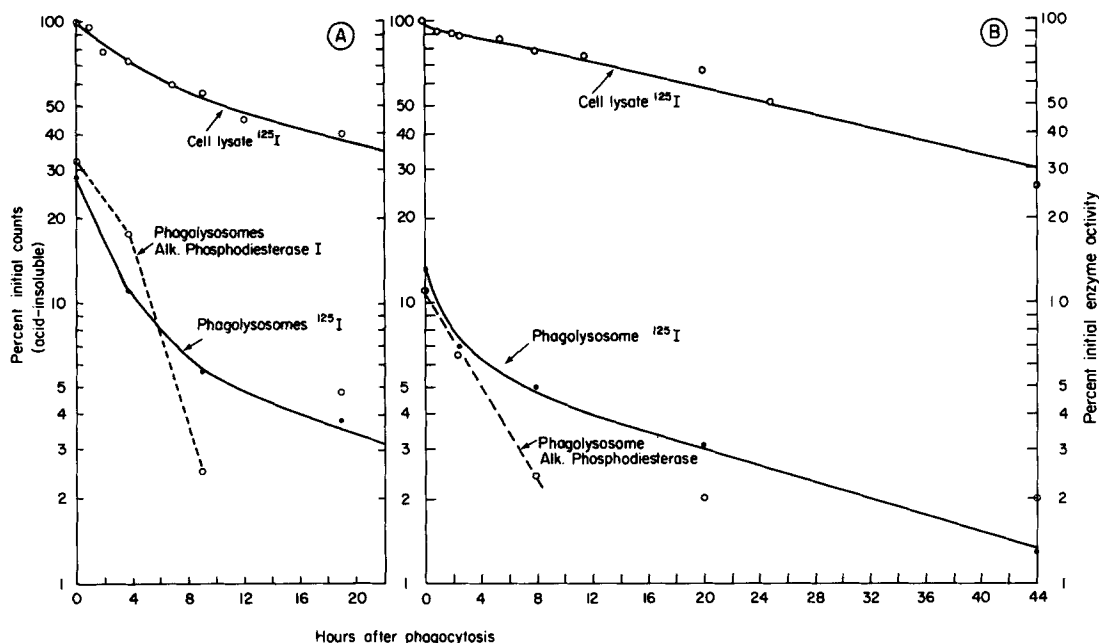


FIGURE 6 Kinetics of label and enzyme activity loss from iodinated L cells and isolated phagolysosomes. TCA-insoluble label remaining cell-associated from 0 to 19 h (A) or 0 to 44 h (B) was plotted semilogarithmically (○—○) as was the percent of the total label found in phagolysosomes (●—●). The actual cpm were corrected to percent of total initial counts. The percent of total alkaline phosphodiesterase I activity recovered in phagolysosomes was also plotted (○--○).

TABLE IV  
Graphical Analysis of Components in Total Cell and Phagolysosome Washout Curves\*

Experiment	Total cell <sup>125</sup> I	Phagolysosome <sup>125</sup> I	Phagolysosome PDase
	%	%	%
A Percent of total	100	29	31
Fast component	23 ( $t_{1/2}$ = 2 h)	73 ( $t_{1/2}$ = 1.75 h)	( $t_{1/2}$ = 4 h)
Slow component	77 ( $t_{1/2}$ = 19 h)	27 ( $t_{1/2}$ = 17 h)	
B Percent of total	100	15.4	11
Fast component	6 ( $t_{1/2}$ = 1 h)	61 ( $t_{1/2}$ = 1 h)	( $t_{1/2}$ = 3 h)
Slow component	94 ( $t_{1/2}$ = 27 h)	39 ( $t_{1/2}$ = 20 h)	

\* See Fig. 6 A and B for curves from which analyses were made.

over 19 h as the cell number per volume increased. The Coomassie blue pattern of the phagolysosomes revealed at least 45 bands ranging in size from 11,000 to >200,000 at 0 h. Changes in the pattern were evident over the washout experiments. Approximately 10 bands exhibited a decrease intensity over time, and 5 an increase. Many bands showed no change (e.g., at 55,000 daltons).

The autoradiographic patterns of both the homogenate and phagolysosomes were quite different from the total protein patterns, reflecting the restricted population of polypeptides available for

iodination. The 0-h homogenate exhibited 10-13 radioactive bands with polypeptides of 140,000, 100,000, 85,000, and 50,000 daltons predominating. The pattern remained unchanged from 0 to 19 h, although the intensity of each band decreased over this period, indicating uniform loss of label. These results were identical to those where total cell radioactivity was analyzed over 48 h in SDS tube gels (Fig. 4). The 0-h radioactivity pattern of phagolysosomes was identical to that of the total cell, demonstrating that when 30% of the plasma membrane was interiorized all labeled membrane

TABLE V  
*Analysis of the Phagolysosomal Fractions at Various Times after Phagocytosis\**

	Hours after phagocytosis	Protein	Acid-insoluble <sup>125</sup> I	PDase	$\beta$ -NAGase	Aryl sulfatase
		%†	%†	%†	%†	%†
Experiment A	0	5.7	28.9	30.6	11.7	11.5
	3.75	6.0	14.4	18.8	40.6	33.0
	9	5.8	10.4	3.8	30.5	20.8
	19	4.9	9.5§	4.1	22.0	15.7
Experiment B	0	4.4	15.4	10.8	7.4	—
	2.5	4.1	7.5	5.9	25.2	—
	8	4.3	6.3	2.5	32.0	—
	20	3.0	5.0	2.8	13.4	—
	44	2.2	4.5	2.1	4.8	—

\* See Fig. 6 A and B for curves of total cell label loss from experiments A and B, respectively.

† Percent of protein, radioactivity, or enzyme activities recovered from the fraction isolated at the times indicated (see Table III for complete analysis of Experiment A, 0-h fractions). Recoveries ranged from 92 to 120%. Values were *not* corrected to the 0-h total cell values.

§ This percentage represents the amount of 19 h total cell label found in the 10%/20% interface fraction. The total acid-insoluble <sup>125</sup>I was 40% of the initial (0 h) total cell label (Fig. 6 A), so 3.8% of the initial total label (9.5 × 40) remained in the phagolysosome at 19 h.

|| Not determined.

proteins were interiorized to the same relative extent as their representation on the plasma membrane. However, the change in the 10%/20% interface labeling pattern from 0 to 19 h was completely different from that of the total cell. Bands at >200,000, 160,000, 120,000, and 100,000 daltons were much less intense by 4 h and absent by 9 h. Other bands decreased more slowly (at 140,000 and 50,000 daltons), and the 85,000 and 12,000 dalton bands decreased in intensity at an even slower rate till at 19 h the 85,000 dalton band was the most prominent on the gel. There was some indication that a second band at 80,000+ daltons appeared at later times (see below). These results suggested that externally disposed membrane proteins that were labeled and randomly interiorized around latex particles were disappearing at quite different rates. The intensity of most of the radioactive bands decreased rapidly. These results agree with the finding of a large fast and a small slow component in the 10%/20% interface decay curves (Fig. 6). Control experiments indicated that there was no preferential loss of labeled bands from the gels during protein staining.

A more quantitative picture of the changes occurring in the phagolysosomes is seen in Fig. 8.<sup>5</sup>

<sup>5</sup> The apparent differences in band and peak positions in Figs. 7 and 8 are due to differences in the polyacrylamide

The labeling patterns of cell homogenates (not shown) from 0 to 19 h were identical in distribution, with only the *amount* of <sup>125</sup>I in each peak decreasing with time. However, this was not the case with the phagolysosomes. The labeling patterns of the cell homogenate and phagolysosomes were identical at 0 h, but by 4 h a change in the distribution of labeled phagolysosomal polypeptides was evident. All peaks had decreased by at least 50% but at slightly different rates, resulting in a different distribution pattern. This differential loss of label continued, confirming the gel autoradiographic results. The relative distribution of radioactivity present in the molecular weight regions indicated was calculated for each time point (Table, Fig. 8). It is apparent that label persisted preferentially in regions C, D, and F over the 19 h. There was no absolute increase in radioactivity in region F (8,000–20,000 daltons) as might have been expected if larger, labeled polypeptides were first degraded to labeled peptides of smaller molecular weight.

A second phagocytosis experiment was extended to 44 h (Fig. 9). The phagolysosomes exhibited a progressive simplification in the radioactivity pattern from 0 to 44 h as most polypeptides disap-

concentration gradients, not variability in the labeling pattern. See legends to Fig. 7 and 8.

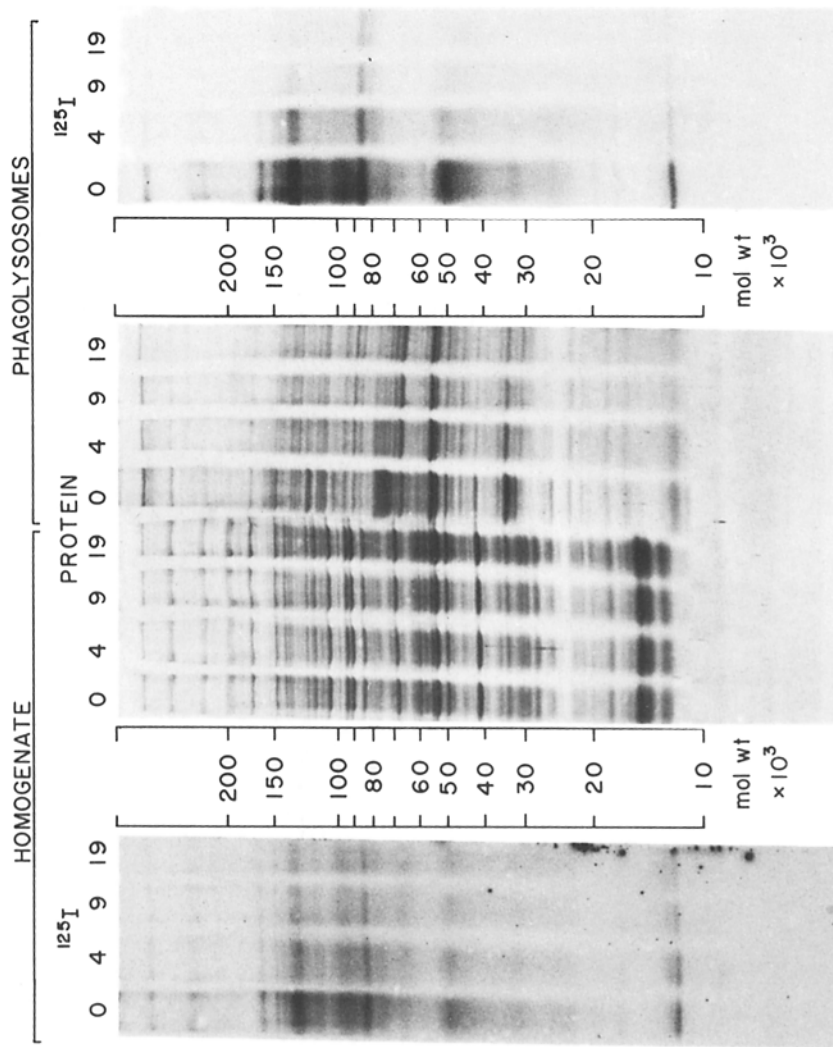
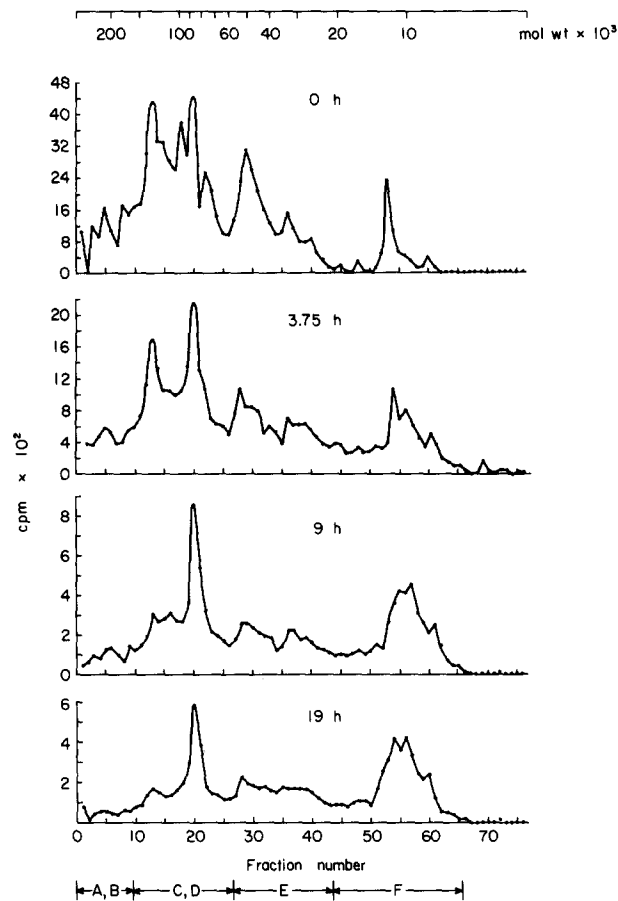


FIGURE 7 SDS-polyacrylamide gel electrophoresis patterns of iodinated cells and isolated phagolysosomes. Ca. 30-40  $\mu$ g homogenate protein (0.01 ml) and ca. 50-60  $\mu$ g phagolysosomal protein (0.4 ml) electrophoresed 8 h at 10 mA, the gel stained for protein with Coomassie blue (center panel), dehydrated, and exposed to X-ray film (outer panels). It was important to electrophorese equal volumes of phagolysosomes or cells from the four time points so direct comparisons could be made, but this meant that the amount of protein applied increased from 0 to 19 h. Homogenate half (left) exposed 21 days and phagolysosome half (right) 4 days. 0, 4(3.75), 9, 19 denote the h after phagocytosis that phagolysosomes were isolated. Resolving gel composition 7.0 to 15% discontinuous acrylamide, 1 mm thick and 12 cm long.



Hours after phagocytosis	Region:			
	A,B	C,D	E	F
0	12.1	47.4	26.6	13.9
3.75	10.0	46.7	22.7	20.5
9	6.0	39.9	22.2	31.8
19	3.5	33.0	25.4	38.0

FIGURE 8 SDS-polyacrylamide gel electrophoresis patterns of isolated phagolysosomes. Equal volumes of phagolysosomes (ca. 100–125  $\mu$ g protein) electrophoresed 12 h at 11 mA, gels cut, and 1.8 mm sections counted. Graphs represent  $^{125}$ I distribution from phagolysosomes isolated 0, 3.75, 9, and 19 h after phagocytosis. The radioactivity in each region, A through F, was summed and expressed as percent of total recovered (lower Table). Gel composition 7.5 to 15% continuous acrylamide, 35:1 A/MBA, 3 mm thick and 13 cm long.

peared relatively rapidly and only a few persisted, predominantly the band at 85,000 daltons. A labeled band of 80,000 daltons was evident at 8 h where previously there was little radioactivity. The intensity of this band remained relatively constant from 8 to 20 h, but was diminished by 44 h. Preliminary phagolysosomal membrane isolation experiments indicated that the long-lived labeled

polypeptides remained associated with the phagolysosomal membrane rather than the content.

The results of gel electrophoresis confirmed the kinetic data on the loss of label from the phagolysosomal compartment. Most of the labeled proteins were degraded rapidly, but at slightly different rates (the fast component). However, label in two molecular weight regions persisted for longer times (the slow component). These regions were at 80,000–85,000 daltons (two bands) and from 8,000 to 15,000 daltons.

## DISCUSSION

### *Fate of Labeled Membrane Proteins*

The enzymatic iodination technique has been successfully used in a study of the flow of membrane and the fate of externally disposed membrane proteins in a eukaryotic cell, the L cell. The surface membrane of living L cells was iodinated,

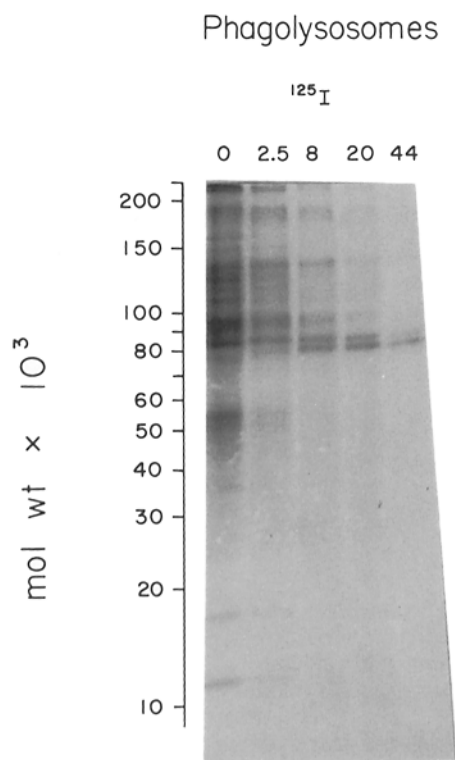


FIGURE 9 Autoradiograph of SDS-polyacrylamide gels of isolated phagolysosomes. Equal volumes of phagolysosomes (ca. 70  $\mu$ g protein) were electrophoresed 8 h at 10 mA, stained for protein, dehydrated, and exposed to X-ray film for 5 days. 0, 2.5, 8, 20 and 44 denote hours after phagocytosis that phagolysosomes were isolated.

after which these cells were cultured in serum-medium with no effect on division rates or other measured cellular functions.

The predominant fate of the labeled membrane proteins proved to be degradation to the level of amino acids. Similar qualitative results have been obtained with iodinated HeLa cells (13). Over 80% of the label lost from the iodinated cells by 47 h in culture was found in the medium as labeled monoiodotyrosine. The degradation was dependent only on cellular processes, not serum factors, since identical results were obtained when cells were cultured in bovine serum albumin. Recovery of most of the MIT in the medium does not indicate that degradation was extracellular. Iodinated human serum albumin or horseradish peroxidase, pinocytosed by cultured mouse peritoneal macrophages, was digested inside lysosomes, and MIT was recovered only in the medium (10, 30). Evidently the intracellular pool size of this non-reutilizable amino acid is quite small.

A variable amount of the label lost from iodinated cells could also be found in the medium, as acid-insoluble radioactivity. The nature of this label was not investigated extensively due to the high concentration of serum proteins in the medium. Cell death, nonspecific sloughing of damaged membrane, virus shedding (21), or specific release all might contribute in part to the label found in the medium. Reports on release of specific proteins from the surface membrane of living cells have generally not established the percentage that this fraction represents of the total membrane turnover nor whether this released material is derived solely from the surface membrane (5, 7, 32).

The loss of label from iodinated L cells occurred at two different rates. From 5% to 20% of the total label was degraded rapidly, with a half-life of 1–3 h. Most of the label, from 80 to 95%, was degraded quite slowly with a half-life of 25–33 h. The degradation of label from both components could be completely inhibited by reducing the temperature to 4°C. Other variables, such as population density, generation rate, number of iodide atoms incorporated, or use of different medium conditions, had no effect. These results suggest that a very stable cell function(s) controls the rate at which plasma membrane proteins are degraded and that only temperature (so far) will affect that cellular mechanism(s).

The forced phagocytosis of latex by iodinated L cells provided a means for studying the intracellu-

lar fate of internalized plasma membrane. The labeled membrane that was interiorized around latex particles was identical to that of the total cell when analyzed on SDS slab gels. These results indicated random internalization of the 15–20 labeled proteins. There was no method for isolation of single latex vacuoles to ascertain whether all the labeled components were present in each vacuole. Internalization of as little as 5% of the total label still resulted in appearance of all the proteins in the isolated phagocytic vacuoles; however, this represents approximately  $80 \mu\text{m}^2$ , a large area in terms of a protein molecule. Others have reported the exclusion of membrane transport sites or ectoenzymes from the membrane internalized during phagocytosis of latex (31, 9). Werb and Cohn (36) found that 5' nucleotidase was internalized to the same relative extent as total cell surface area and that a maximum of 60% of the membrane could be internalized in a phagocytic cell, the mouse peritoneal macrophage. It is possible, of course, that any one labeled protein (e.g., a transport site) represents such a small percent of the total label that its exclusion during internalization would not be detected.

Latex-filled vacuoles isolated at the earliest times after phagocytosis contained lysosomal enzyme activities and thus were phagolysosomes. The membrane surrounding the latex was both lysosome and plasma membrane derived. Enzyme histochemistry might reveal whether every vacuole, or only a few, contained acid hydrolase activity at 0 h. Others have reported this method as a plasma membrane isolation procedure, but did not measure the extent to which lysosomes had already fused with the latex-filled vacuole (12).

The kinetics of label loss in the phagolysosomes revealed a very fast rate of degradation ( $t_{1/2} \sim 1-2$  h) of 70–80% of the internalized radioactivity. It is unlikely that a major portion of labeled membrane was cycled back to the cell surface after internalization because (a) the latex is not digestible and so the internalized membrane surface area remained largely unchanged in size in the phagolysosome, and (b) the amount of label lost from the phagolysosomal compartment in the first 2.5–4 h was slightly less than label lost from the total cell (Table IV and Fig. 6). The inactivation of alkaline phosphodiesterase I activity in the phagolysosome accounted for the loss of enzyme activity in the whole cell, again arguing against membrane recycling. There is not yet conclusive proof that lysosomal enzymes degraded the labeled peptide segments of the externally disposed membrane

proteins; however, L cells contain lysosomal proteinases and peptidases capable of degrading proteins to the level of amino acids (29). Since only the exposed peptide segments of most externally disposed membrane proteins were iodinated, and the conformation of most membrane proteins is unknown, it is not known whether the unlabeled portions of these proteins were also degraded rapidly.

The rapid loss of most of the label ( $t_{1/2} = 1-2$  h) as well as the activity of one plasma membrane enzyme ( $t_{1/2} = 3$  h) agreed with the results of Werb and Cohn (36) on the inactivation of 5' nucleotidase after entry into the lysosomal compartment. Soluble proteins sequestered in secondary lysosomes after pinocytosis have been reported to be degraded with longer half-lives, from 6 h when iodinated human serum albumin is used (10), to 30 h when a cationic protein such as lysozyme is used.\* More rapid degradation of membrane protein may reflect a greater instability of these proteins, well-known for 5' nucleotidase (Werb and Cohn, 1972).

A small portion (20–30%) of the labeled membrane proteins was degraded slowly, with a half-life of approximately 20 h. Some of this label undoubtedly represented continuing endocytosis of labeled plasma membrane and entry into the lysosomal compartment. Alkaline phosphodiesterase I activity was slightly increased in the phagolysosomes at 19 h compared to 9 h (Fig. 6), suggesting continuing endocytosis. Incubation of L cells during this time with NaF, an inhibitor of pinocytosis, should reduce the enzyme activity found, as was reported for 5' nucleotidase activity (36). Others have reported finding newly pinocytosed material in secondary lysosomes of L cells (11). However, the slow rate component also reflected the presence of a slowly degraded group of labeled polypeptides found at 85,000 and 8,000–15,000 daltons in SDS gels. These bands would never have become so prominent if all labeled proteins were degraded with very similar half-lives. In addition, a new, radioactive polypeptide was detected between 4 and 8 h and was quite long lived, thus contributing to the slow rate component. This polypeptide may represent a hydrolysis product of a larger, labeled protein which was resistant to further digestion, analogous to the 72,000 mol wt red cell membrane component *a* after pronase hydrolysis (14).

\* Z. A. Cohn, unpublished observations.



### *Other Studies of Membrane Protein Turnover*

Other investigators have studied plasma membrane turnover with a variety of techniques. As yet, no one has attempted to purify a plasma membrane protein as was done for a number of endoplasmic reticulum proteins (2, 26). Rather, total membrane components were examined. Most of the radiolabels employed were reutilizable, so the half-lives obtained were maximum values. In addition, the membrane preparations isolated were not well characterized. Using double isotope techniques (1), Dehlinger and Schimke (8) found a general correlation between the relative degradation rates of rat liver plasma membrane proteins and the molecular size of the polypeptides. Since rat liver is composed of at least two cell types with quite different functions, and since the parenchymal cell has at least two specialized areas of surface membrane (the junctional complex and the bile front canaliculi), plasma membrane preparations must be quite heterogeneous and would make data on turnover difficult to interpret.

The use of homogeneous populations of tissue culture cells eliminated the problem inherent in the isolation of plasma membrane from organs, but the purity of the plasma membrane prepared has been a continual problem in studying membrane turnover.

L-cell membrane turnover was studied using a number of different internal labels, [ $^{14}\text{C}$ ]leucine, [ $^{14}\text{C}$ ]glucosamine, and [ $^{14}\text{C}$ ]glucose (33). The last label is rather nonspecific and no attempt was made to identify the species labeled. Pulse-chase experiments were performed and the recovery of label was not quantitated on a volume basis but on the basis of whole membrane ghosts counted in a hemocytometer. In addition, the purity and yield of the membrane preparations were not established at each time point. For these reasons the results obtained are difficult to interpret, yet they appear to contradict those we have found. The membrane from growing L cells exhibited a  $7 \pm 2\%$  loss of incorporated lipid, protein, or carbohydrate per generation (20 h). Differences in membrane turnover were reported between growing and nongrowing L cells, which we did not observe, but we were not able to maintain L cells at high densities longer than 16 h without extreme acidification of the medium and loss of cell viability.

Nachman et al. (25) found a 7–8-h half-life of [ $^{14}\text{C}$ ]leucine in the plasma membrane of rabbit alveolar macrophages. This cell type is known to

endocytose at a rapid rate which is dependent on the serum concentration (6, 30). An internalization rate of 8% of cell surface area per hour followed by rapid degradation of *all* the labeled plasma membrane proteins, regardless of orientation in the membrane, would account for this degradation rate. A more rapid endocytic rate with partial recycling and partial degradation would also fit the data.

Others have studied the kinetics of regeneration of plasma membrane components after exposure of intact cells to hydrolytic enzymes (for review see 23; also references 17, 22, 24, 28, 35). In each study about 6–10 h were required for full recovery of the parameter being measured (sialic acid residues, H-2 antigen, virus receptor, cholesterol exchange capacity, etc.). Without additional data it is impossible to conclude whether these rates reflect a normal membrane protein synthetic rate or an altered rate in response to a damaged cell surface. Phagocytosis in macrophages and polymorphonuclear leukocytes stimulates an increased turnover of specific phospholipids (see review, 19), but no net synthesis at the time of phagocytosis. Werb and Cohn (36) have demonstrated a net increase in plasma membrane constituents in the mouse peritoneal macrophage (5' nucleotidase, phospholipids, cholesterol) starting approximately 6 h after phagocytosis. The amount of new membrane synthesized was proportional to the amount of membrane interiorized around latex particles, suggesting that interiorization of membrane constituents and their subsequent degradation is tightly coupled to the cellular processes involved in membrane synthesis.

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