

MEMBRANE FLOW DURING PINOCYTOSIS

A Stereologic Analysis

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ABSTRACT

HRP has been used as a cytochemical marker for a stereologic analysis of pinocytic vesicles and secondary lysosomes in cultivated macrophages and L cells. Evidence is presented that the diaminobenzidine technique (*a*) detects all vacuoles containing enzyme and (*b*) distinguishes between incoming pinocytic vesicles and those which have fused with pre-existing lysosomes to form secondary lysosomes.

The HRP reactive pinocytic vesicle space fills completely within 5 min after exposure to enzyme, while the secondary lysosome compartment is saturated in 45–60 min. The size distribution of sectioned (profile) vacuole diameters was measured at equilibrium and converted to actual (spherical) dimensions using a technique modified from Dr. S. D. Wicksell.

The most important findings in this study have to do with the rate at which pinocytosed fluid and surface membrane move into the cell and on their subsequent fate. Each minute macrophages form at least 125 pinocytic vesicles having a fractional vol of 0.43% of the cell's volume and a fractional area of 3.1% of the cell's surface area. The fractional volume and surface area influx rates for L cells were 0.05% and 0.8% per minute respectively. Macrophages and L cells thus interiorize the equivalent of their cell surface area every 33 and 125 min.

During a 3-h period, the size of the secondary lysosome compartment remains constant and represents 2.5% of the cell volume and 18% of the surface area. Each hour, therefore, the volume and surface area of incoming vesicles is 10 times greater than the dimensions of the secondary lysosomes in both macrophages and L cells. This implies a rapid reduction in vesicle size during the formation of the secondary lysosome and the egress of pinocytosed fluid from the vacuole and the cell. In addition, we postulate that membrane components of the vacuole are subsequently recycled back to the cell surface.

Pinocytosis involves the formation of a membrane-bound vesicle from the cell surface, trapping within it fluid and solutes from the extracellular environment. In most cells, these incoming pinocytic vesicles fuse with acid-hydrolase-containing granules or lysosomes. These events have been studied largely in relation to macromolecular and small

particulate markers occupying the content of the incoming vesicle (e.g., 9, 15, 40, 41). In these instances, interiorized materials undergo digestion and/or sequestration within the lysosomal apparatus. Very little else is known about pinocytic vesicles. We lack quantitative information on their surface areas and volumes, especially in relation to

the area and volume of the whole cell. We do not know the composition of the vesicle membrane, the factors which lead to fusion with lysosomes, and the fate of the vesicle surface after such fusion. Finally, we do not fully understand the function of this widespread cellular activity in higher organisms.

We have been interested in obtaining quantitative information on the pinocytic activity of cultured mammalian cells. Our initial studies (40, 41) were concerned with the rate of uptake of solutes, since it has been generally assumed that pinocytosis serves some nutritional role. We found that the glycoprotein enzyme, horseradish peroxidase (HRP), was a useful marker. In both macrophages and L cells, cell-bound HRP can be localized cytochemically to the vacuolar system. Quantitative studies with a sensitive enzymatic assay demonstrated that it is taken up in the fluid phase without prior adsorption to the cell surface. Uptake is directly proportional to the concentration of HRP in the culture medium over a large range (0.05–2.0 mg/ml), proceeds continuously with time, and is highly temperature dependent. Finally administration of HRP did not appear to alter the ongoing pinocytic activity of cultured cells (41). Using the HRP model, we conclude that 10^6 macrophages and growing L cells pinocytose about 10^{-4} ml of culture medium each hour, i.e., the rate of uptake of fluid phase solutes is 0.01% of the administered solute load/milliliter. Moreover, the uptake and degradation rates of HRP remain constant for days in cultures of both cell types. In this study, we use stereologic techniques to focus on the quantities of vesicle membrane and fluid interiorized during pinocytosis and on their subsequent fate within the cell.

MATERIALS AND METHODS

Cells

Primary monolayer cultures of resident mouse peritoneal macrophages were obtained as described (6) and maintained for 24 h before use in 35-mm plastic Petri dishes in 20% fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N.Y.) medium 199 (Grand Island) supplemented with penicillin. L cells, a continuous line of mouse fibroblasts, were maintained as monolayers in 60-mm plastic Petri dishes in 5% FCS-minimal essential medium (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.) supplemented with penicillin and streptomycin, and were studied during the rapid log phase of growth.

Horseradish Peroxidase (HRP)

The Type II preparation of Sigma Chemical Co. (St. Louis, Mo.) was used and generally administered to cells as a 1 mg/ml solution in culture medium. Levels of HRP uptake were quantitated with an enzymatic assay as previously outlined (40, 41). The Graham and Karnovsky diaminobenzidine (DAB) (24) technique was used to localize cell-bound enzyme, also as previously described (40, 41).

Cytochemical analysis was often performed on cells pulsed for very brief periods with HRP (0–180 s). Culture dishes were taken from the CO_2 incubator and placed in a thin layer of water in a 37°C water bath. At time 0, medium containing HRP at 37°C was added. To terminate the HRP exposure and pinocytic activity quickly (within 15 s), the medium was aspirated, and the entire dish was passed through three 250-ml beakers of ice-cold phosphate-buffered saline (PBS), followed by 2.5% glutaraldehyde in 0.1 M cacodylate buffer as fixative. Zero-time cultures were treated in an identical fashion, i.e., HRP was added to the cells and then aspirated immediately.

Other Pinocytic Markers

The rate of HRP uptake was compared with that of ^3H -sucrose, another fluid-phase pinocytic marker for tissue culture work (46). ^3H -sucrose, 2 Ci/mol, was obtained from Amersham/Searle Corp., Arlington Heights, Ill. and administered at a concentration of 10^{-6} M (2×10^6 cpm/ml) in tissue culture medium. As in assays for HRP uptake, the monolayers were washed five times in medium 199 and then left at 37°C for $\frac{1}{2}$ h before lysis in 0.5 ml of 0.05% (vol/vol) Triton X-100 (Sigma Chemical) in water. 100 μl of cell lysate was then counted in 10-ml Aquasol scintillation fluid (New England Nuclear, Boston, Mass.) using a Nuclear-Chicago Mark II scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) operating at 43% efficiency. This sucrose uptake assay is relatively insensitive and prolonged exposures (up to 6 h) are required for reliable data.

A colloidal thorium dioxide (Thorotrast, Fellows-Tagar Div., Fellows Mfer. Co., Inc., Detroit, Mich.) preparation was also used as a pinocytic marker for electron microscope studies. The cells were exposed to a 1–30 dilution (vol/vol) of colloid in tissue culture medium for 2h.

Sampling Procedure for Stereologic Studies

Random samples were obtained as follows. Each monolayer culture was fixed and processed as a cell pellet in agar according to the technique of Hirsch and Fedorko (28). If the cells are scraped from the monolayer after the initial glutaraldehyde fixation, a cell pellet is obtained in which the cells are evenly distributed and randomly oriented. Three to six pieces from each pellet were

sectioned and collected on large holed grids. Micrographs of most cells on a single section were then taken and examined at a final magnification of 10,000 with appropriate graticules. Generally 40–100 micrographs were analyzed per culture, corresponding to sections through 100–300 cell profiles (profiles in which the cell nucleus was present). Individual or pairs of cultures were examined, since it was previously established that the absolute level of pinocytic activity varies little in replicate cultures (40, 41). Reproducibility was assessed by doing experiments on two or more occasions with different batches of cells.

Cell Dimensions

The volume of cells was measured from the cell diameter using the relation $V = \pi D^3/6$. Cells were allowed to assume spherical shapes after removal from monolayer culture. L cells become spherical after trypsinization from culture dishes by a standard procedure (41). Macrophages were more difficult to remove. A useful procedure for this work was to chill monolayers in ice-cold PBS. When some of the cells began to round up (15–30 min), the cells were scraped from the dishes with a policeman. More than 90% are trypan blue negative, and most are spherical. On four different occasions, we measured the diameters of 200 cells at a magnification of 1,000 using a calibrated micrometer eyepiece.

Cell protein determinations were made with a modified Lowry procedure (35) using hen egg-white lysozyme as standard (Worthington Biochemical Corp., Freehold, N.J.). The same preparations that were used to measure cell diameter were counted in a hemocytometer and analyzed for protein content. The amount of cell protein in these samples was identical to that obtained if the monolayers were simply lysed outright in 0.05% Triton X-100 in water.

Cell surface areas were determined by stereologic techniques, since their outlines were so irregular (microvilli, ruffles, blebs, etc.) The surface area per cell is found by dividing the surface to volume ratio, S_v , by the number of cells per unit volume, N_v , where volume refers to the total volume of cells and embedding plastic. For any micrograph, S_v , the ratio of cell surface area to specimen volume, is given by $2P_L$, where P_L is the number of intersections a grid of equally spaced parallel lines makes with the cell surface, per unit length of line (19, 34, 48). Our test grid had 19 lines, 25-cm long and 1-cm apart, and our final magnification was 10,000. N_v , the number of cells per unit volume of specimen, is given by the fraction of test points falling on cells times the volume represented by each point, divided by the volume of individual cells. Our grid had 475 test points, 1-cm apart so that each point at $\times 10,000$ represents $1 \mu\text{m}^3$. A sample calculation is as follows. The 475 cm of test line made an average of 105 intersections with the macrophage cell surface per micrograph, so that S_v was $2 \times$

$105/475 \mu\text{m}^2$ cell surface/ μm^3 specimen. Of 475 test points per micrograph, an average of 110 fell on cells. Since the volume of a macrophage is $395 \mu\text{m}^3$, the number of cells per unit volume of specimen is $110/475 \times 1/395$. The surface area per cell is then $2 \times 105 \times 395/110$ or $754 \mu\text{m}^2$ for this set of micrographs.

In some instances, we labeled the surface of macrophages with a presumptive nonpenetrating marker, according to Bernhard and Avrameas (2). A $100 \mu\text{g/ml}$ solution of concanavalin A (con A) (Miles Yeda, Yeda Research and Development Co., Ltd., Rehóvoh, Israel) was attached to the surface of glutaraldehyde-fixed cells over a 15-min period, the bound Con A was then made visible by its ability to bind a $50 \mu\text{g/ml}$ solution of HRP, and this was followed by staining with the DAB procedure.

Vacuole Dimensions

Vacuoles involved in pinocytosis of HRP were identified by their content of cytochemical reaction product in otherwise unstained sections. The criteria for distinguishing incoming pinocytic vesicles and secondary lysosomes (collectively referred to as vacuoles) will be described in the Results section. Since most are circular in shape, their diameters can be measured and a frequency distribution plotted. The latter is a distribution of profile or sectioned diameters, as opposed to vacuole, or spherical, diameters. Wickcell (52) has devised a procedure for obtaining (a) the distribution of vacuole sizes that gives rise to the observed profile data, and (b) the average vacuole diameter, surface area, and volume. The exact procedure we used was that of S. Brodie and B. W. Knight (manuscript in preparation) of The Rockefeller University, who have slightly modified the Wickcell analysis and programmed it for computer use. The section thickness, $0.08 \mu\text{m}$, was included in the computation.

Two approaches were taken to determine the area and volume of the entire pinocytic vesicle and lysosome compartment. The first and easiest approach is to use a grid of test points and lines (19, 34, 49). This provides a vacuole volume fraction, given by the frequency with which test points fall on vacuole vs. cell, and a surface area fraction, given by the frequency with which test lines intersect vacuole vs. cell surface. These fractions were multiplied by the cell surface area and volume (see Materials and Methods) to get the absolute size of the vacuole compartment. A second method is to multiply the average vacuole area and volume (see preceding paragraph) by the number of vacuoles per unit volume of cell, N_v . N_v is obtained from the number of vacuole profiles per unit area of section. This is converted to a number per unit volume by dividing by " $\bar{D} + t$ ", where \bar{D} is the average vacuole diameter and " t " the section thickness, $0.08 \mu\text{m}$ (18, 19, 34, 48).

RESULTS

Cellular Dimensions (Table I)

Macrophages and L cells assume spherical shapes when removed from a monolayer into suspension culture. The diameters of each cell type fell in a narrow distribution, the mean being $9.1 \pm 0.8 \mu\text{m}$ for macrophages and $15.0 \pm 1.1 \mu\text{m}$ for L cells. With the formula $\pi D^3/6$, we obtained average volumes of $385 \pm 40 \mu\text{m}^3$ and $1,765 \pm 130 \mu\text{m}^3$ respectively.

The protein content of cells in suspension was measured next, since quantitative data on HRP uptake have generally been measured on a per unit of protein basis (40, 41). 10^6 macrophages have $80 \pm 12 \mu\text{g}$ of protein, using lysozyme as standard in the Lowry procedure, while 10^6 L cells had $455 \pm 20 \mu\text{g}$ at the stage of growth studied. The macrophage value is higher than that previously reported (40), as the latter data was based on erroneous cell counts.

The surface areas of the two cell types were determined by stereologic techniques. The averages were 825 ± 80 for macrophages and $2,100 \pm 245$ for L cells. These results are 3.3 and 3.0 times that which would have been obtained had these cells been assumed to be smooth spheres with areas of πD^2 .

The identification of the macrophage surface is complicated by the presence of peripheral lacunae, i.e., vesicles in the peripheral cytoplasm which appear to be intracellular but in fact are connected to the extracellular space. In routine sections, many of these can be distinguished from actual intracellular pinocytotic vesicles by their irregular shapes. To assess the extent of error introduced by these lacunae, we labeled the cell surface with con A and peroxidase, according to Bernhard and Avrameas (2). In four specimens, less than 4% of the cell surface intersections passed through labeled spherical lacunae, i.e., structures that in

routine section would have been considered as intracellular pinocytotic vesicles. For practical purposes, then, the macrophage plasma membrane can be identified in routine sections.

Most of our work on the dimensions of endocytic vacuoles, and the kinetics with which they are formed, has been performed on macrophages. The macrophage data will be presented in detail followed by some comparative observations on mouse L cells.

HRP as a Marker for Distinguishing Pinocytotic Vesicles and Secondary Lysosomes

As previously noted (40, 41), cytochemical localization of pinocytosed HRP yields vacuoles with reaction product in two types of distribution. In one, only a peripheral rim of cytochemical reactivity is seen, adjacent to the inner aspect of the vacuole membrane, while in the other, the entire granule is filled with product. Two lines of morphologic evidence were obtained to show that the former represent recently formed pinocytotic vesicles, while the latter are secondary lysosomes, i.e., vesicles which have fused with existing acid-hydrolyase-filled structures.

Kinetic studies first revealed that macrophages exposed for a brief period to HRP (3 min or less), contain vacuoles with only the peripheral rim of reactivity (Fig. 1). These presumptive pinocytotic vesicles were entirely intracellular since binding of HRP to the cell surface is not demonstrable (40, 41, and Figs. 1-5 and 7). Longer periods of exposure (beginning at 5 min) are required to produce the dense reactive granule (Fig. 2). If macrophages are exposed to HRP (60 min) and then cultured in HRP-free medium (30 min) (to chase enzyme from the incoming vesicle compartment), then the peripheral rim type of reactive

TABLE I
Dimensions of Macrophages and L Cells

Parameter	Macrophage	L cell
Diameter (μm)	9.1 ± 0.8	15.0 ± 1.1
Volume (μm^3)	385 ± 40	$1,765 \pm 130$
Surface area (μm^2)	825 ± 80	$2,100 \pm 245$
Protein ($\mu\text{g}/10^6$ cells)	80 ± 12	455 ± 20

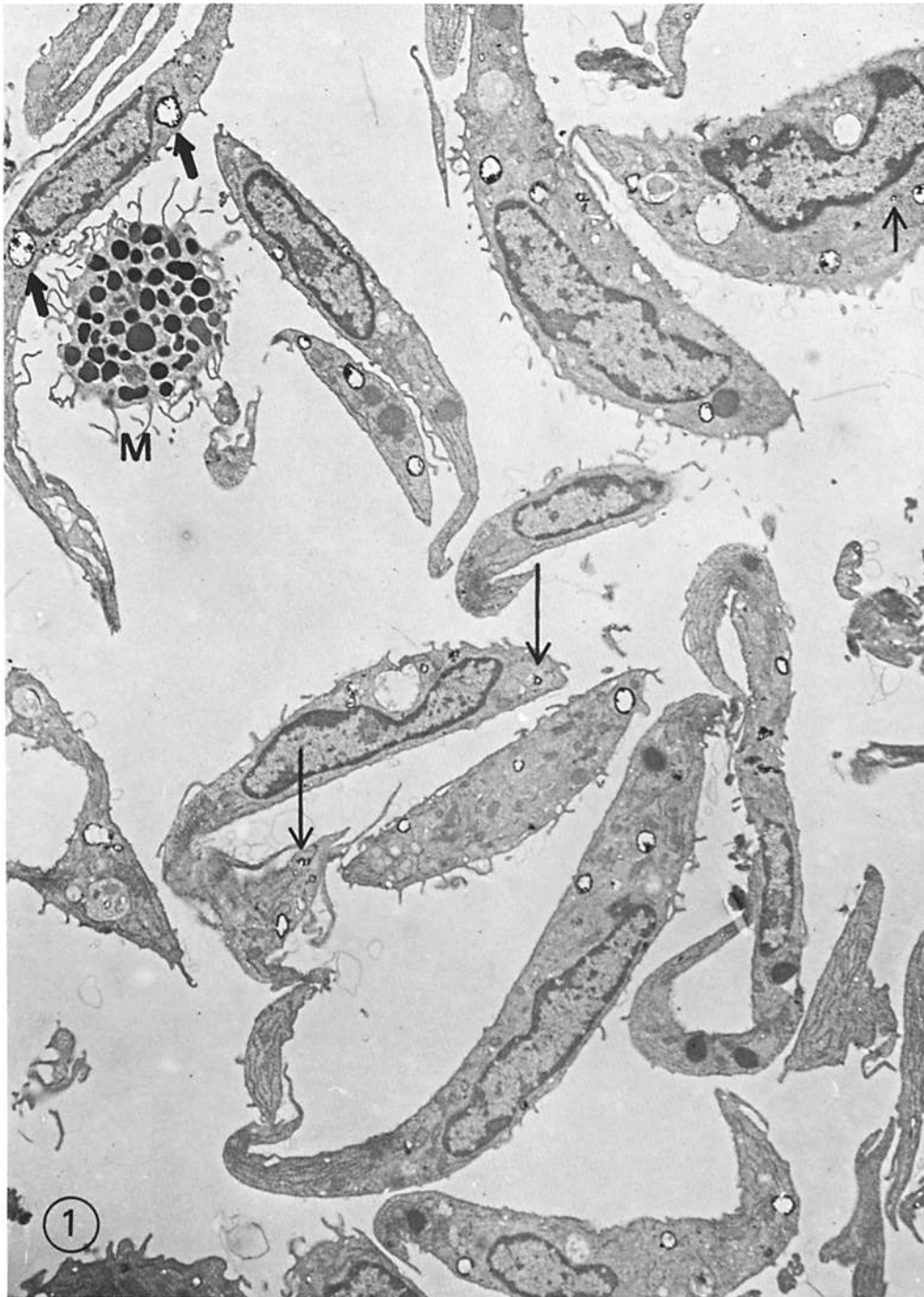


FIGURE 1 A section through a cell pellet of macrophages exposed for 3 min to 1 mg/ml of HRP. The cell population is uniform with respect to cell type and content of reaction product. Mast cells (*M*) are infrequent. Incoming pinocytic vesicles have a peripheral rim of cytochemical reactivity. They are spherical in shape and exhibit a continuous distribution of profile diameters. The largest in this section are 0.8 μm (short arrows, top left) and the smallest (e.g., long arrows) are 0.1–0.2 μm . After this brief exposure to enzyme, reactive vacuoles are distributed throughout the cytoplasm. This micrograph is magnified 5,000 \times which is one-half the magnification used in this work.

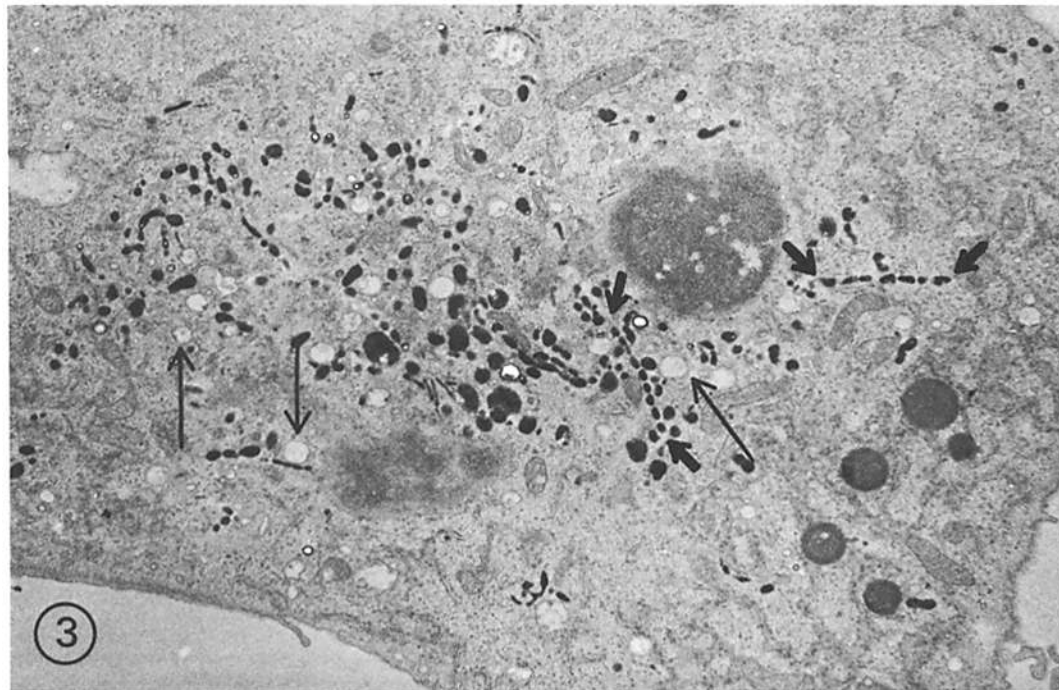
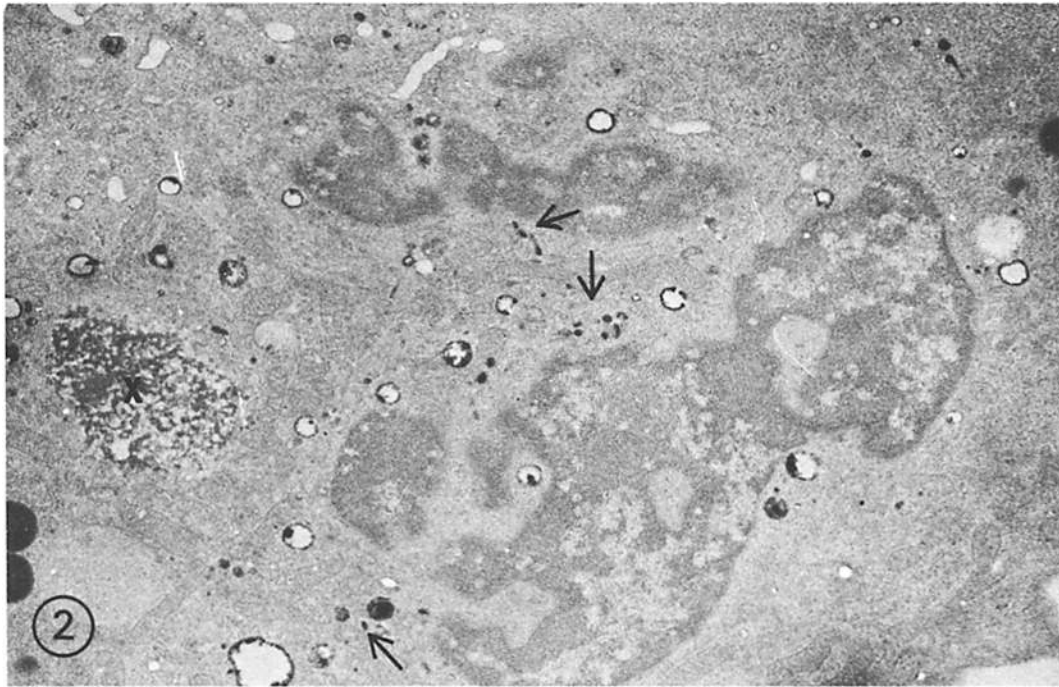


FIGURE 2 A section in the plane of the cell monolayer of a macrophage exposed 10 min to 1 mg/ml HRP. Most reactive vacuoles have a peripheral run of reactivity and are accumulating in the perinuclear region. Some secondary lysosomes replete with reaction product (arrows) have formed. Their numbers increase greatly with further HRP exposure. The inclusion, labeled X, may be a large vacuole with HRP. However these infrequent structures are seen in cells not exposed to enzyme and may be degenerating phagocytosed cells. $\times 6,500$.

FIGURE 3 A section similar to that in Fig. 2 except that the culture was exposed to HRP for 1 h and then chased in HRP-free medium for $\frac{1}{2}$ h. Presumptive pinocytotic vesicles (long arrows) now lack reaction product. Many reactive secondary lysosomes are present in the perinuclear region. Some are aligned in long rows (enclosed by short arrows) which extend out into the cell pseudopods. $\times 7,500$.

vacuoles are rare while the dense structures are abundant (Fig. 3).

A second line of evidence involved double labeling of cells, first treating them with colloidal thorium dioxide and then exposing them for 1 h to the cytochemical tracer, HRP. The rationale here is that the colloid would be chased from pinocytic vesicles into secondary lysosomes during the 1-h HRP exposure, so that pinocytic vesicles would contain only HRP, and secondary lysosomes both markers. In fact, little or no thorium dioxide was visualized in several hundred putative incoming HRP-reactive vesicles (i.e., those with a peripheral rim of reactivity), whereas most vacuoles filled with HRP-reaction product exhibited colloid as well (Figs. 4 and 5). We rarely found profiles with colloidal thorium and no HRP. If Thorotrast and HRP were given to cells simultaneously, colloid and enzyme reaction product were found in both types of HRP-reactive vacuole. We have not yet succeeded in using cytochemical techniques to localize an acid hydrolase (e.g., acid phosphatase) to the granules labeled with HRP. However, previous studies have shown that secondary lysosomes marked with acid-phosphatase reaction product are similar in structure and distribution to those containing dense HRP reactivity (9). For the remainder of this study, we refer to vacuoles with a peripheral rim of cytochemical reactivity as pinocytic vesicles, whereas those with a dense content are called secondary lysosomes. Although presumptive pinocytic vesicles are often larger than lysosomes (see below), the distinction between the two types of vacuole is made solely on the basis of the distribution of cytochemical reaction product vs. size.

The Sensitivity of the Cytochemical Technique

The other important advantage of HRP as a marker solute is that the DAB technique appears to detect all incoming pinocytic vesicles. Previous studies (40, 41) established that HRP was pinocytosed in the fluid phase of incoming vesicles, so that increasing the concentration of enzyme in the culture medium should simply increase its concentration in the pinocytic vesicle. If the cytochemical assay is fully sensitive, there should exist some concentration of HRP at which the number and dimensions of HRP reactive vesicles reaches a plateau; raising the concentration of enzyme in the culture medium (hence vesicles) should not change

their detectability even though the absolute amount of interiorized HRP would increase. This was indeed observed in macrophages exposed for 2.5 min to 0.1, 0.5, 1.0, and 2.0 mg/ml HRP (Table II). There was no significant difference in the number, fractional surface areas, and fractional volumes of pinocytic vesicles in cells given the three higher concentrations of enzyme, while all three parameters decreased significantly at 0.1 mg/ml (lines 1-3, Table II). Control data showed that the overall cellular dimensions were not altered by the different concentrations of HRP, viz, the surface area of the cells and the size of the cell profiles (lines 4 and 5, Table II). The DAB procedure must therefore detect all incoming pinocytic vesicles which form from culture medium containing 0.5 mg/ml HRP or more. Further supporting evidence will be presented below.

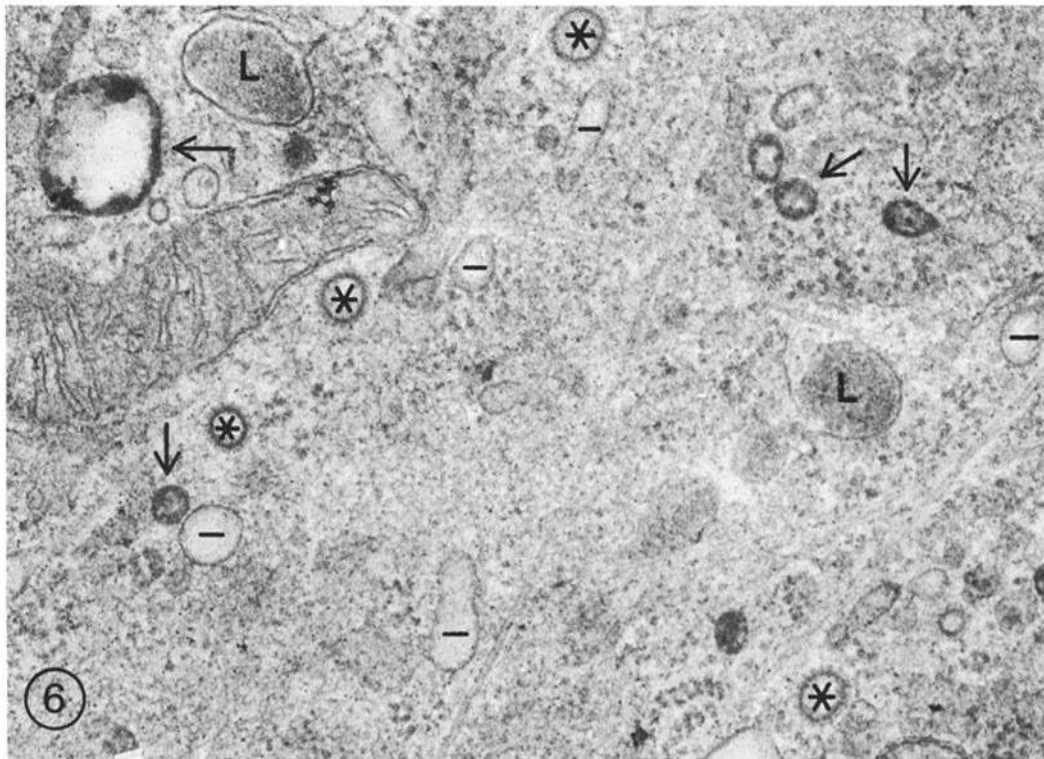
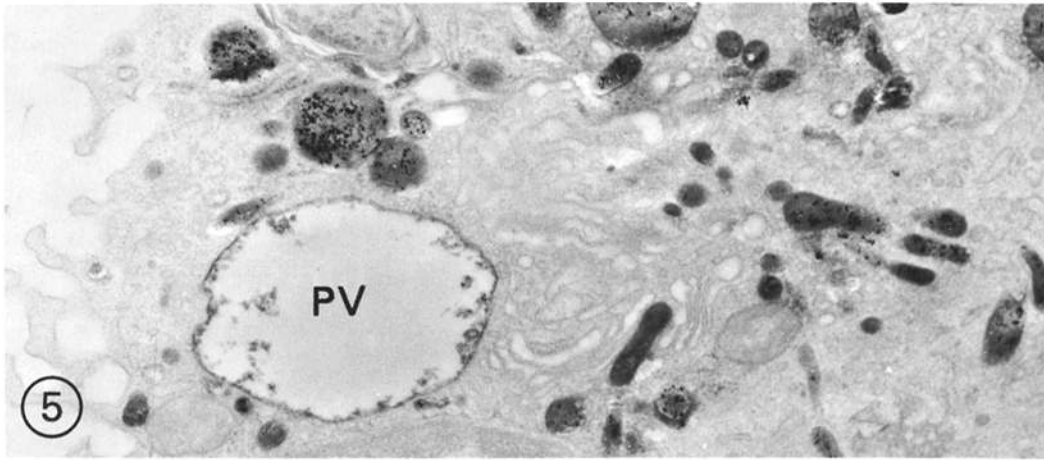
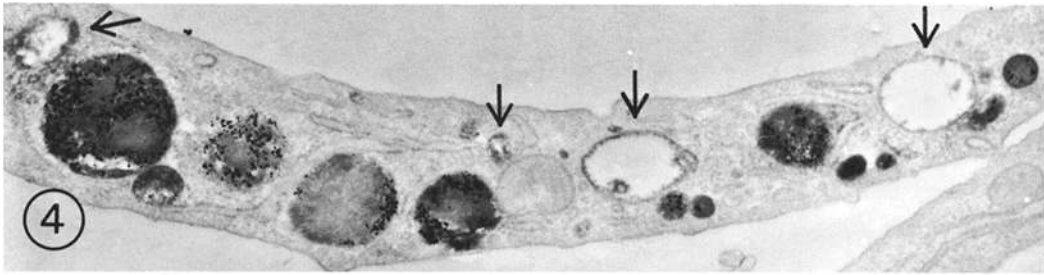
The Size Distribution of Pinocytic Vesicles and Secondary Lysosomes

The diameters of newly formed pinocytic vesicles were measured in cells exposed for 30, 60, 150, 180, and 300 s to 1 mg/ml HRP (Table III). Some 10-20% of the vesicle profiles were not circular, so that an estimate of their equivalent circular diameter was made. With time, the relative frequency of larger profiles increased. We suspect this is due to fusion among incoming pinocytic vesicles. The composite distribution for 5,703 vesicle profiles, representing the cumulative data in Table III, has been plotted (Fig. 7 A).

The distribution of profile diameters can be converted to actual vesicle diameters using a procedure of S. Brodie and B. W. Knight (manuscript in preparation, modified from Wicksell [52]). Data for the mean actual vesicle diameter, area, and volume are presented in Table IV. It is noteworthy that if vesicles must be greater than 0.3 μm to be resolved by phase contrast microscopy, then at least 84% of the incoming vesicles (30 s data) would not be detectable.

Macrophages, like other cells, contain a population of small, 0.1 μm , coated vesicles, i.e., vesicles in which tiny spikes or bristles project from the surface into the cytoplasm. These coated vesicles predominated along the dish surface of cultured cells. At either cell surface, however, they lacked HRP reaction product (Fig. 6).

Longer exposures of macrophages to HRP results in the appearance of a large population of HRP-containing secondary lysosomes (Figs. 2 and



3). The frequency size distribution of 4,850 sectioned (profile) lysosome diameters, after 60 min of HRP, has been obtained (Fig. 7 B). When converted to actual vacuole diameters, this distribution gives an average lysosome diameter, area, and volume of 0.136 μm , 0.108 μm^2 , and 0.0074 μm^3 . 92% of the lysosomes have a diameter of 0.30 μm or less, in cells cultivated under those conditions.

The Kinetics of Pinocytic Vesicle Formation

Since the DAB technique faithfully identifies incoming pinocytic vesicles, we exposed cells for 0–5 min to 1 mg/ml HRP to quantitate the initial rate of vesicle fluid entry. This was determined first by measuring the fraction of test points falling on vacuole vs. cell after varying times in HRP. A sample field that was analyzed is shown in Fig. 1.

TABLE II
Sensitivity of the Diaminobenzidine Technique

Measurement	Dose of HRP in culture medium mg/ml			
	0.1	0.5	1.0	2.0
1. No. vesicles/points on cell	0.109 ±0.026	0.199 ±0.062	0.215 ±0.048	0.229 ±0.022
2. Vesicle/cell intersections	0.051 ±0.024	0.093 ±0.018	0.106 ±0.014	0.103 ±0.013
3. Vesicle/cell points	0.0062 ±0.0022	0.0113 ±0.0037	0.0126 ±0.0021	0.0123 ±0.0038
4. Cell intersections/cell points	0.902 ±0.099	0.950 ±0.041	0.922 ±0.090	0.921 ±0.073
5. Cell points/no. cell profiles	46.5 ±5.8	40.0 ±5.6	40.9 ±2.5	47.0 ±7.1

Measurements (\pm standard deviation) were made of cytochemically reactive pinocytic vesicles after a 2.5-min exposure to varying concentrations of HRP. Line 1 gives the numerical density of vesicles per unit area of cell in section; lines 2 and 3 the surface area and volume fractions of vesicles relative to the whole cell; line 4 relates the surface area of the cells to their volume; and line 5 reflects the absolute size of the cells.

FIGURE 4 A doubly labeled macrophage, loaded for 2 h with colloidal thorium dioxide (the electron-dense particles), washed, and then exposed for 1 h to HRP. The rationale is that most of the ThO_2 would move into secondary lysosomes during the last hour in HRP, while HRP should be found in both pinocytic vesicles and lysosomes. In fact, the presumptive, HRP-reactive, pinocytic vesicles (arrows) lack colloid, while vacuoles with a dense content of HRP reaction product contain ThO_2 . $\times 20,000$.

FIGURE 5 Another doubly labeled macrophage. Again the presumptive pinocytic vesicles with HRP (PV) contain few electron-dense thorium dioxide particles, while dense HRP-reactive granules have abundant colloid. Even though this is a thin section labeled briefly with both markers, it is evident that ThO_2 and HRP accumulate in the same lysosome compartment. $\times 23,500$.

FIGURE 6 A macrophage pulsed 5 min with HRP and sectioned close to the dish surface, where coated vesicles (*) are frequent. Unlike the other micrographs, this has been stained with lead and uranyl salts to better visualize the bristles or spikes on the cytoplasmic aspect of coated vesicles. The latter do not exhibit HRP. Several other types of smooth surfaced vacuoles are present in this field. Some have reaction product (arrows), some lack any content whatsoever, (—), while others have an electron-dense content but no HRP (L). The latter presumably are lysosomes that have not yet fused with incoming reactive vesicles. $\times 56,000$.

TABLE III
Frequency Distribution of Pinocytic Vesicle Diameters

Diameter of pinocytic vesicle in section	Time in HRP (s)				
	30	60	150	180	300
μm					
<0.1	17.5	13.2	16.1	12.5	10.0
0.1-0.2	36.9	33.6	36.7	35.8	32.9
0.2-0.3	20.2	17.9	14.7	16.4	16.2
0.3-0.4	12.1	12.9	12.1	12.0	13.6
0.4-0.5	4.7	6.9	6.7	7.1	8.3
0.5-0.6	4.4	4.6	4.2	5.3	5.4
0.6-0.7	1.5	3.0	3.6	4.0	4.5
0.7-0.8	2.0	1.7	2.4	2.9	2.3
0.8-0.9	0.2	1.0	1.3	1.3	2.4
0.9-1.0	0.5	0.5	0.7	0.6	1.6
> 1.0	0.2	0.7	1.5	1.5	2.6
Total no. vesicles in sample	410	1,297	1,352	871	1,773

Macrophages were exposed to 1 mg/ml HRP for brief periods at 37°C. The experiments were terminated quickly (about 15 s) by washing in cold PBS followed by fixation.

TABLE IV
Average Dimensions of HRP-Reactive Vacuoles

Average vacuole dimensions	Macrophage pinocytic vesicles after varying times in HRP (s)					Macrophage secondary lysosomes	L-cell pinocytic vesicles
	30	60	150	180	300		
Diameter (μm)	0.202	0.228	0.216	0.238	0.266	0.136	0.202
Area (μm^2)	0.196	0.251	0.247	0.282	0.351	0.109	0.162
Volume (μm^3)	0.0143	0.0207	0.0224	0.0259	0.0356	0.00741	0.00847

The data on sectioned (profile) diameters (Table III, and Figs. 7 and 13) were converted to distributions of actual (spherical) dimensions.

The volume fraction occupied by HRP reactive vesicles was found to increase linearly at a rate of 0.43% of the total cell volume/minute (Fig. 8 A). Since individual macrophages have an average volume of 395 μm^3 , there is an influx of 1.7 μm^3 /cell/min. After 5 min, the volume fraction occupied by reactive vesicles plateaus (Fig. 10).

Another stereologic approach to obtain the volume fraction is to utilize data on the average volume per vesicle (Table IV) and multiply it by the number of vesicles per unit volume, N_V . During the first 3 min in enzyme, the number of reactive vesicle profiles/unit area of section increases at a rate of 0.062 profiles/ μm^2 /min (Fig. 8 B). This

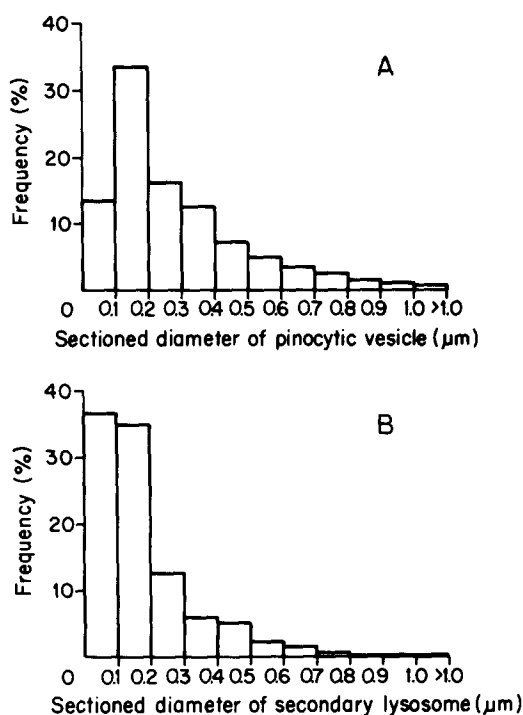


FIGURE 7 (A) Frequency distribution of the sectioned diameters of pinocytotic vesicles, i.e. HRP-reactive vacuoles with a peripheral rim of reactivity. The histogram is from the cumulative data on 5,703 profiles in Table II, i.e. for cells exposed $\frac{1}{2}$ –5 min to HRP. (B) The sectioned diameters of 4,850 secondary lysosomes, i.e. vacuolar profiles having a dense content of cytochemical reaction product after a 60 min exposure to HRP.

value may be an underestimate due to vesicle fusion, e.g., for the first time-point (30 s), the rate is 0.086 profiles/ μm^2 cell section/min. The rates of profile formation can be converted to N_V , the number of vesicles per unit volume, by dividing by $\bar{D} + t$ where \bar{D} is the mean vesicle diameter and t , the section thickness. N_V is then 0.206 vacuoles/ μm^3 cell/min for the composite 3 min data (\bar{D} is 0.220) and 0.304 for the 30 s data (\bar{D} is 0.202). The volume fractions of pinocytotic vesicles/minute is N_V times the average vesicle volume, and is 0.43% of the cell volume/minute for both the composite 3 min data and the 30 s data (average volumes of 0.0207 and 0.0143, respectively).

These rates of fluid influx mean that some 25% of the total (cytoplasm and nucleus) macrophage volume would be taken in each hour. A similar value can be obtained by an entirely independent nonstereologic procedure. Since HRP is pinocy-

tosed in the fluid phase, the rate of fluid interiorization can be determined by dividing the absolute amount of HRP uptake, with an enzymatic assay, by the concentration of HRP in the culture medium. At a concentration of 1 mg/ml, enzyme is pinocytosed continuously by macrophages (Fig. 9). The rate is 1.2 ng, or 1.2×10^{-6} ml per μg /protein/h. This corresponds to a rate of 1.4×10^{-6} ml since cells inactivate HRP continuously at a rate of 10% h (40, 41). Since 10^4 macrophages have 80 μg protein (Table I), one obtains a value of $1.2 \mu\text{m}^3$ solution taken in per cell per min.

Another concern about the high rate of fluid uptake may be that it bears little relation to the ongoing pinocytotic activity of cells not exposed to HRP. This too seems unlikely. Uptake of HRP is continuous; even data taken with brief HRP exposures (15 min) fits the straight line relating HRP uptake with time (Fig. 9). Also the uptake of another pinocytotic marker, ^3H -sucrose (47), occurs at the same rate as HRP, whether HRP is present simultaneously or not (Fig. 9).

We next measured the surface area of incoming vesicles in relation to the total cell surface area, as given by the relative frequency with which a grid of test lines intersects vacuole vs. cell surface. During the first minutes of exposure, the surface area of HRP reactive vacuoles increases at a rate of 3.1% of the cell surface area/minute (Fig. 8 C). Since the surface area of the macrophage is 825 μm^2 , the rate of formation of vesicle membrane is 26 μm^2 /cell/min. Alternatively, the area of membrane pinocytosed per minute, using the 30 s data on vesicle profile sizes, is given by the number of vesicles per unit volume, times the area per vesicle, times the volume of the cell, i.e., 0.304 vesicles/ $\mu\text{m}^3 \times 0.196 \mu\text{m}^2$ /vesicle $\times 395 \mu\text{m}^3$ per cell or 24 μm^2 /cell.

The Fate of Interiorized Pinocytotic Vesicles

It has long been apparent that incoming pinocytotic vesicles fuse with preformed lysosomes. In fact, our previous kinetic and morphologic data implied that all pinocytosed HRP undergoes inactivation and degradation within lysosomes (40, 41). This process of fusion with lysosomes can also be followed cytochemically in cells exposed for prolonged periods (5–180 min) to 1 mg/ml of enzyme.

HRP-containing secondary lysosomes, i.e., membrane-bound granules filled with cytochemical reaction product, first appear 5 min after exposure to enzyme. The earliest reactive granules

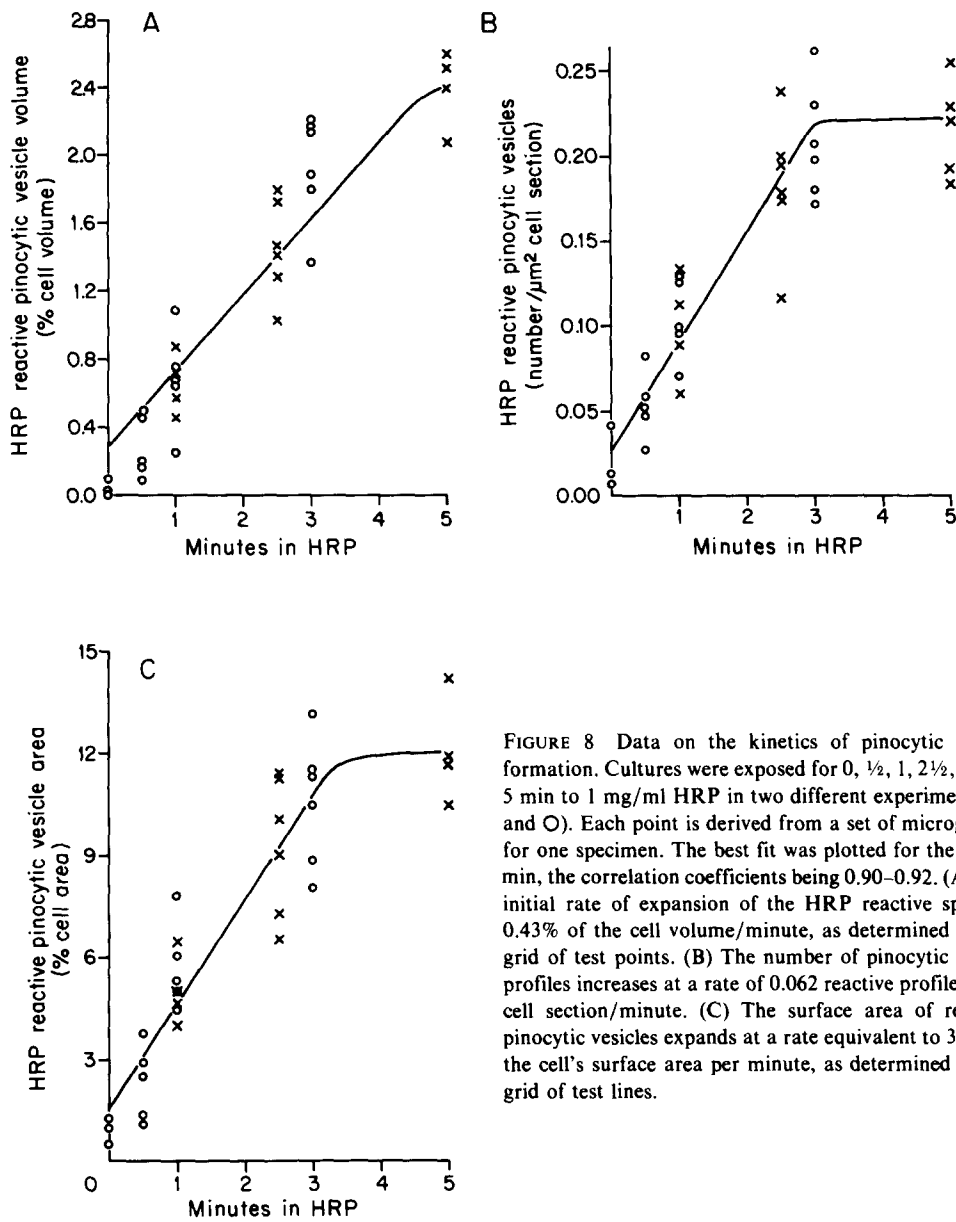


FIGURE 8 Data on the kinetics of pinocytic vesicle formation. Cultures were exposed for 0, 1/2, 1, 2 1/2, 3, and 5 min to 1 mg/ml HRP in two different experiments (\times and \circ). Each point is derived from a set of micrographs for one specimen. The best fit was plotted for the first 3 min, the correlation coefficients being 0.90–0.92. (A) The initial rate of expansion of the HRP reactive space is 0.43% of the cell volume/minute, as determined with a grid of test points. (B) The number of pinocytic vesicle profiles increases at a rate of 0.062 reactive profiles/ μm^2 cell section/minute. (C) The surface area of reactive pinocytic vesicles expands at a rate equivalent to 3.1% of the cell's surface area per minute, as determined with a grid of test lines.

are most readily apparent in the Golgi region (Fig. 2). The number of HRP-reactive lysosomes increases progressively for some 40 min, and then plateaus at a level of 0.60 profiles/ μm^2 cell section over the remaining period of study (up to 3 h in all) (Fig. 10 A). With an average lysosome diameter of 0.136 μm (Table IV, calculated from Fig. 7 B), we find that the number of vesicles per unit volume is 2.77. An average cell, 395 μm^3 in volume, therefore contains 1,100 lysosomes. The surface area

and volume of secondary lysosomes also increases during the 5–45 min period in HRP and then plateaus. Using a grid of test lines and points we find that the plateau values are such that lysosomes have a fractional surface area equivalent to 18% of the cell surface area and a fractional volume of 2.5% (Fig. 10 B and C). Using a value of 1,100 lysosomes with average dimensions of 0.109 μm^2 area and 0.0074 μm^3 volume (Table IV derived from Fig. 7 B), the fractional surface

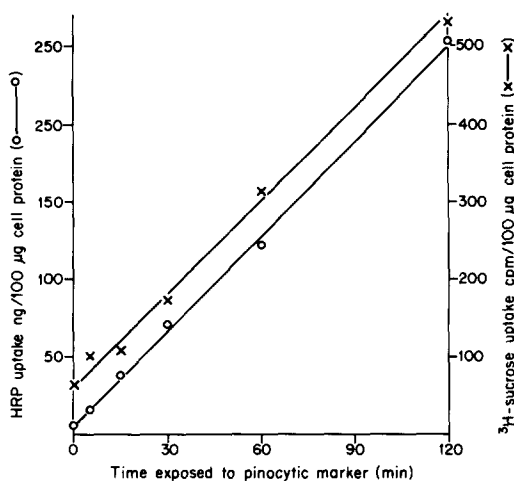


FIGURE 9 The kinetics of solute uptake using HRP (O—O) and ³H-sucrose (x—x) as fluid-phase markers. Both are taken up continuously at a rate of 0.012% of the administered load/hour/100 μg protein. The HRP assay is sensitive enough so that brief exposures to enzyme give suitable data. The sucrose assay is less sensitive; the data presented are actually the initial part of a 6 h exposure to label.

area is 14.5% and volume is 2.1%, in agreement with data obtained with test grids of lines and points.

Throughout the time-period studied, i.e., after 5 min in HRP, the dimensions of the HRP-reactive pinocytic vesicle space remains constant (Fig. 10 A-C). The number of profiles/μm² plateaus at 0.21 (Fig. 10 A) yielding an N_V of 0.61 vesicles/μm³ (using the average diameter from the 5-min vesicle dimensions in Tables III and IV). Cells 395 μm³ in volume therefore have 240 vesicles of which 70% are 0.30 μm in diameter or less. The fractional volume of the pinocytic vesicle space is 2.5% (using point counting - Fig. 10 B) or 2.2% (using N_V times the average volume per vacuole - 5 min data in Table IV). The fractional surface area is 12.5% (using intersection counting, Fig. 10 C) or 10.2% (using the average vacuole area, 5 min data in Table IV).

We conclude that during the first 5 min of exposure to HRP, enzyme fills the pinocytic vesicle compartment of macrophages, and then gradually (5-45 min) is distributed within a pre-existing lysosomal space whose dimensions probably remain constant throughout the period of this experiment. To test this, a pulse chase experiment was performed in which cells were exposed to HRP

for 15 min, and then chased in HRP-free medium for 45 min. At this time, most presumptive pinocytic vesicles lacked HRP reactivity, and cytochemical product was limited to dense secondary lysosomes. However, the dimensions of the reactive vacuoles were similar to that seen after a 60-min continuous exposure (Table V), even though quantitatively they contain one-quarter as much HRP.

The critical feature of these data, as evident in Fig. 10, is that much more vesicle surface and fluid is entering the cell than can be accounted for subsequently in the lysosomal compartment. Even if all the lysosomes are newly formed each hour (which is unlikely), pinocytosis brings in 1,535 μm² of surface and 102 μm³ of fluid into a lysosomal space having an area of 150 μm² and a volume of 10 μm³. Whereas solute (HRP) is accumulating continuously (Fig. 9), the data indicate that membrane and fluid must rapidly leave the lysosome compartment.

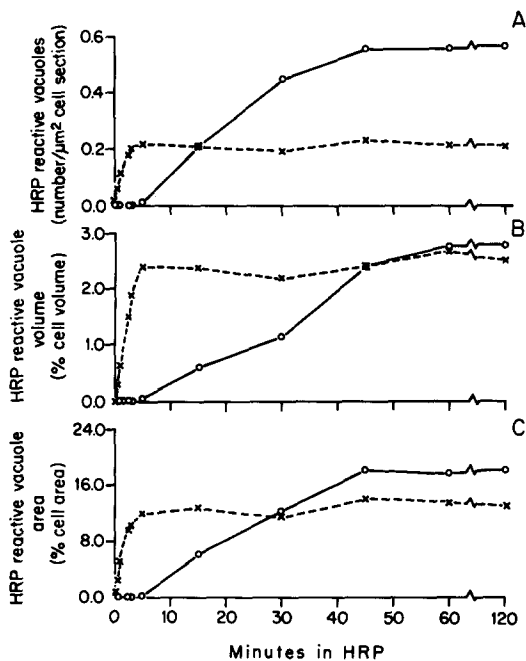


FIGURE 10 The kinetics of appearance of HRP reactive pinocytic vesicles (x—x) and secondary lysosomes (O—O) after varying periods in enzyme. The number (A), volume (B), and area (C) of reactive pinocytic vesicles plateaus after 5 min in enzyme, while the lysosome compartment takes 45 min to fill. Though not shown here, the plateau values persist after 3 h in HRP as well.

TABLE V
Pulse Chase of HRP in Macrophages

	< 60 min continuous HRP	15 min in HRP, then 45 min without HRP
HRP reactive pinocytic vesicles		
Number/ μm^2 cell section	0.208	0.004
Relative surface are (% cell)	12.1	0.7
Relative volume (% cell)	2.65	0.3
HRP reactive secondary lysosomes		
Number/ μm^2 cell section	0.563	0.541
Relative surface area (% cell)	15.7	13.6
Relative volume (% cell)	2.53	2.38

Stereologic analysis of cells exposed continuously to 1 mg/ml HRP (60 min) or to a 15 min pulse followed by a 45 min chase. The chase greatly diminishes the number of HRP-reactive pinocytic vesicles, but has little effect on the reactive secondary lysosome compartment.

Stereologic Analysis of Pinocytosis in L Cells

The stereologic findings on HRP-reactive vacuoles were qualitatively similar in both L cells and macrophages. Incoming L cell pinocytic vesicles were identified by their peripheral rim of cytochemical reactivity, and secondary lysosomes by their dense content (41 and Fig. 11). Pinocytic vesicles were spherical in shape, and again, coated vesicles were devoid of HRP (Fig. 12). The frequency distribution of sectioned vesicle diameters was similar to that seen in macrophages, except that large profiles (0.5 μm or more in diameter) were rare (Fig. 13). The average vesicle diameter, area, and volume are 0.20 μm , 0.16 μm^2 , and 0.0085 μm^3 (Table IV).

The volume and surface area of HRP reactive pinocytic vesicles and secondary lysosomes were measured with a test grid in cells exposed for varying times to 1 mg/ml HRP (Table VI). The initial rate of expansion of the pinocytic vesicle space was 0.05% of the cell volume/min, which is 0.9 μm^3 /min for cells having a volume of 1,765 μm^3 (Table I). This rate is similar to that predicted from the level of HRP solute uptake (41), i.e., cells take up 0.11 ng HRP/ μg cell protein/h from a 1 mg/ml solution. At this stage of growth, 1 μg of protein corresponds to 2.2×10^8 cells, so that the fluid uptake per cell per min by this method is 0.8 μm^3 . The rate of interiorization of pinocytic vesicle surface corresponds to 0.8% of the cell surface/min or 1,100 μm^2 /h. The average L cell forms 100

vesicles per minute. This value times the average vesicle area and volume (Table IV) gives fractional areas and volumes identical to that obtained with a test grid.

HRP-reactive secondary lysosomes first appeared in L cells after a 5-min exposure to HRP. Their number, area, and volume then increased progressively with time (Table VI). We did not attempt to establish that these values reach a maximum after 1 h, as they do in macrophages. However, the dimensions of the lysosomal compartment are again much smaller than that which apparently enters the cell in pinocytic vesicles. After 1 h, pinocytosis brings in 54 μm^3 of fluid and 1,100 μm^2 of surface area, but the corresponding dimensions of the vacuolar system (pinocytic vesicles and secondary lysosomes) are just 20 μm^3 and 220 μm^2 .

DISCUSSION

HRP as a Marker for the Stereologic Analysis of the Vacuolar System

In previous studies (40, 41), we have used HRP as a marker to follow the uptake and fate of solutes in the vacuolar system of cultivated mouse macrophages and L cells. In this paper, we employ the same marker to obtain quantitative data on the uptake and fate of the fluid and surrounding membrane of pinocytic vesicles. The suitability of HRP for this sort of work derives from two

features of the Graham and Karnovsky DAB technique used to localize intracellular enzyme (24).

(a) Under appropriate conditions, the cytochemical procedure detects all pinocytic vesicles containing HRP. The best evidence was as follows. Since HRP is taken up as a solute in the fluid

phase (40, 41), we could use our previous quantitative data on enzyme uptake to predict the rate at which the pinocytic vesicle space would expand. Thus the volume of fluid interiorized is the amount of solute taken in, divided by the concentration of solute in the fluid phase. These values are $1.2 \mu\text{m}^3/\text{macrophage}/\text{min}$ and $0.8 \mu\text{m}^3/\text{L cell}/\text{min}$.

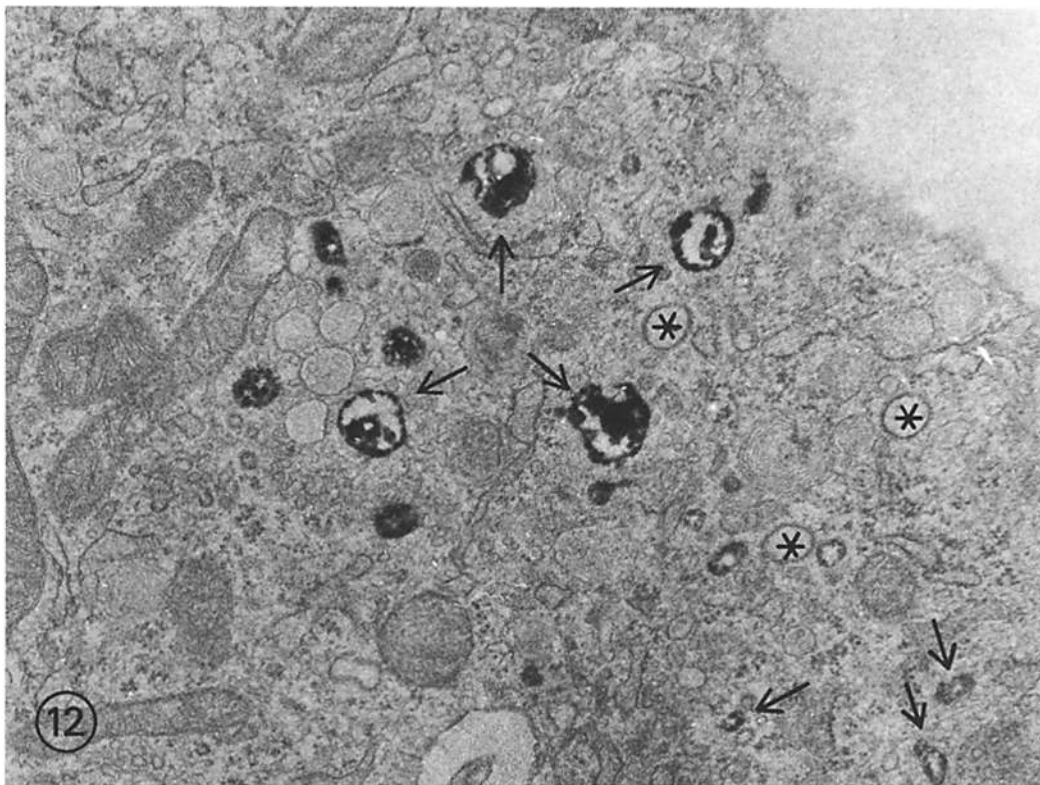
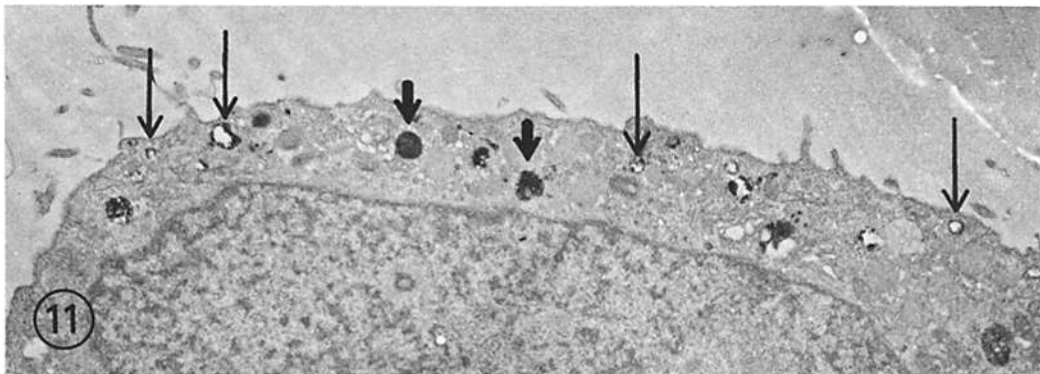


FIGURE 11 An L cell exposed 15 min to 1 mg/ml HRP contains pinocytic vesicles of varying diameter (long arrows) and secondary lysosomes (short arrows). $\times 14,000$.

FIGURE 12 Coated vesicles (*) in L cells also do not contain HRP whereas smooth surfaced ones do (arrows). $\times 31,500$.

Direct measurement, by point counting, of specimens pulsed for brief periods with HRP showed that the rate of expansion of the HRP-reactive pinocytic vesicle space was in fact 1.7 and 0.9 $\mu\text{m}^3/\text{min}$ for macrophages and L cells, respectively. In macrophages, the somewhat higher value obtained with the stereologic method may be in error, e.g., a small error in the cell diameter measurement would produce a large error in the result; or incoming HRP-containing vesicles may fuse with preexisting HRP-negative ones, produc-

ing a misleading larger, reactive vacuole; or cells may shrink during fixation.

Further evidence that the DAB procedure was fully sensitive was the observation that increasing the concentration of HRP in the fluid phase beyond 0.5 mg/ml (to 1.0 and 2.0 mg/ml) did not alter the detectability of reactive pinocytic vesicles, even though the amount of enzyme in these vesicles increases linearly with increasing concentration of HRP in the culture fluid (40, 41). The cytochemical reaction therefore behaves as if it is saturated with enzyme at the concentration of 1 mg/ml employed in this work.

(b) The distribution of reaction product within HRP-containing vacuoles enabled us to distinguish whether that vacuole had undergone fusion with preexisting lysosomes to form a secondary lysosome. In the macrophages and L cells we studied, newly arising pinocytic vesicles exhibit reaction product peripherally along the inner aspect of the surrounding membrane, while HRP-containing, secondary lysosomes have a uniform dense content of reaction product. This distinction was made first on the basis of kinetic studies. Vesicles with peripheral reactivity were the only positive vacuoles found after brief exposures (5 min or less) to HRP, and quickly became nonreactive when the cells were placed in HRP-free medium. In addition, double-label experiments demonstrated that the presumptive secondary lysosomes were the only HRP-reactive vacu-

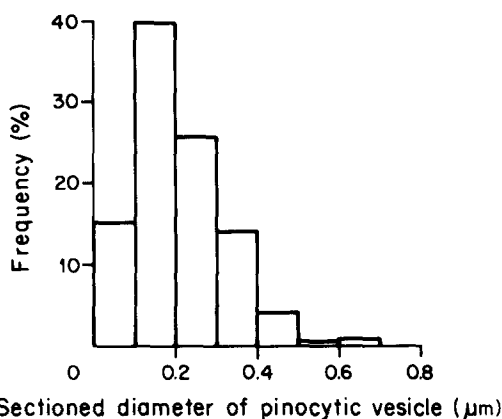


FIGURE 13 Frequency distribution of profile diameters of L cell pinocytic vesicles formed after 1 and 5 min exposures to HRP. The histogram is similar to that seen in macrophages (Fig. 7) except that large profiles (0.5 μm or more) are rare.

TABLE VI
Stereologic Analysis of L-Cell Vacuoles

Parameter	Time in 1 mg/ml HRP (min)				
	1	5	10	15	60
No. vacuoles/μm^2 cell section					
Pinocytic vesicles	0.017	0.078	0.087	0.100	0.089
Secondary lysosomes	0.000	0.001	0.030	0.052	0.217
Fractional vacuole volume (% cell volume)					
Pinocytic vesicles	0.05	0.25	0.33	0.34	0.32
Secondary lysosomes	0.00	0.00	0.11	0.33	0.89
Relative vacuole surface area (% cell surface area)					
Pinocytic vesicles	0.8	4.5	4.4	4.1	4.0
Secondary lysosomes	0.0	0.2	2.2	3.0	6.5

Growing L-cell monolayers were exposed to 1 mg/ml HRP for varying times. Three specimens from each culture were examined by stereologic techniques. The cells in all cultures had identical volumes and surface area. Pinocytic vesicles were identified by their peripheral rim of cytochemical reactivity while secondary lysosomes were replete with reaction product.

oles containing colloidal thorium dioxide, another secondary lysosome marker. We are not certain why the cytochemical reaction produced such a different distribution of reaction product in the history of a pinocytic vesicle. We suspect that HRP within incoming vesicles becomes bound to the surrounding membrane during fixation and that reaction product does not diffuse far from this site. In contrast, HRP may become distributed throughout the content of fixed secondary lysosomes, either because its concentration is so high due to vacuolar shrinkage (see below), or because the enzyme binds to the internal content of the vacuole during fixation.

Another important advantage of the system we have studied is that HRP does not alter the ongoing pinocytic activity of cultured cells. We detect no obvious morphologic changes after HRP administration, and the uptake of other soluble markers is not changed by exposure to the enzyme (41 and this study). The cells we have chosen to study are themselves quite stable in that they pinocytose HRP at similar rates for several days *in vitro*, and are capable of degrading it with similar kinetics at all times.

The Dimensions of Pinocytic Vesicles

The first application of HRP as a marker solute was to identify the incoming vesicle. One possible difficulty with previous morphologic studies was that the marker employed was either bound to the cell membrane, as in the case of colloids that absorb to the cell surface, or was not cleared from the extracellular space. In both circumstances, the marker may appear to be in an intracellular vesicle but, in fact, may lie in a surface invagination. Because HRP is not absorbed to the cell surface, cytochemically reactive vesicles in washed cultivated cells must be pinocytic in origin.

Using HRP as a marker, we found that incoming vesicles were spherical in shape and exhibited a continuous range of diameters. In macrophages, the range was 0.05–1 μm , while in L cells, vesicles larger than 0.5 μm were rare. Many if not all of the large vesicles in macrophages may be the result of fusion of vesicles of smaller sizes. The continuous spectrum of vesicle diameters argues against the presence of two distinct classes of incoming pinocytic vesicles (4, 53), corresponding to so-called micro (about 1,000 \AA in diameter) and macro (visible by light microscopy) pinocytosis. We also could not show that coated vesicles, i.e., 0.1 μm

vesicles exhibiting bristles or spokes on the cytoplasmic aspect of their limiting membrane, were involved in the pinocytosis of HRP. These vesicles were first noted to be abundant in cells interiorizing large amounts of protein (37) and appeared to be involved in the uptake of exogenous tracers (22). Conceivably, coated vesicles have an endocytic role in macrophages and L cells, but we could not detect it. For example, what appears to be a coated vesicle in section may be in the process of invagination, so that HRP was simply washed away before fixation. Another possibility is that these vesicles may lose their coating shortly after interiorization, during 15–30 s it took to initiate fixation. It is possible, however, that coated vesicles serve other functions than fluid-phase pinocytosis in cultivated cells, e.g., they could have a secretory function, or they may be involved in the recycling of pinocytosed membrane (see below).

The Influx of Pinocytic Vesicle Fluid and Membrane

A striking feature of pinocytic vesicles is the large amounts of fluid and membrane which they bring into the cell. By direct stereologic measurements, we find that each hour, macrophages and L cells interiorize the equivalent of 26% and 3% of their cell volume, and 190% and 48% of their surface area, respectively. These fractional influxes can be converted to absolute levels of fluid and membrane interiorization if the cell volume is known. At this time, we cannot measure the volume of monolayer cells directly, and so our data derive from cells removed from the monolayer. We have confirmed that the stereologic findings for L cell monolayers apply as well to L cells given HRP in suspension cultures (our unpublished data).

Lewis (33) in his initial description of pinocytosis in cultivated mammalian cells, pointed out that macrophages may take in one-third their volume each hour (though the cell volume data on which the estimate was based seem implausible). The dimensions of the pinocytic vesicle compartment relative to those of the whole cell were not subsequently appreciated except for the work of Bowers and Olszewski in *Acanthamoeba Castellanii* (3). These authors measured the rate of pinocytic fluid uptake. They then estimated that the equivalent of 5–50 times the organism's surface area was interiorized, depending on the actual size of the incoming vesicle and assuming the amoeba had a smooth surface.

With respect to the high rate of fluid and solute influx during pinocytosis, Lewis stated, "The importance of this phenomenon in cellular metabolism and in the economy of intercellular fluids is almost self-evident (33)." But the relevance of a high fluid and solute influx to the cellular economy is far from obvious. The current view is that most cells are capable of transporting fluid, electrolytes, and small molecular weight nutrients by efficient transport systems operating at the cell surface. Pinocytosis of fluid and solutes would therefore seem redundant. When macromolecular solutes are interiorized, they too are digested to small molecular weight components in the lysosomal space (8, 15, 16, 17, 40). It is likely that the electrolyte composition of vesicle fluid, being a sample of the extracellular fluid, contains high concentrations of the very ions the cell is actively (i.e., spending metabolic energy) trying to exclude, such as sodium and calcium. It is therefore not clear yet in what way the content of the pinocytic vesicle is required for cellular function.

The function of the high rate of membrane interiorization during pinocytosis is also uncertain. It would allow for a rapid uptake of anything binding to the cell surface. This in turn may be important in removing certain ligands from the extracellular space, in terminating signals generated by ligands at the cell surface, or simply in enhancing the uptake and delivery of certain materials, e.g., vitamins and hormones bound to macromolecular carriers. Adsorptive pinocytosis is a much more efficient method of solute uptake when compared to fluid-phase pinocytosis, especially at low concentrations of ligand (e.g., 31, 46). For example, 1,000,000 macrophages interiorize about 1/10,000th of an administered solute concentration/hour by fluid-phase pinocytosis. If a receptor exists and it binds 1% of the administered solute concentration, then membrane interiorization would bring in 2/100 of the administered load, since twice the cell surface enters in this time.

At issue here is the composition of the vesicle membrane in relation to the plasma membrane from which it derives. Is the entering membrane a representative or a selective sample of the plasma membrane? The latter possibility has been raised by studies in lymphocytes where the binding of appropriate ligands, like anti-immunoglobulin reagents, leads to pinocytosis of the surface-binding site for the ligand (immunoglobulin) without altering the expression of other binding sites, e.g.,

histocompatibility antigens (43). The question of bulk vs. selective uptake of plasma membrane during endocytosis has only been studied in relation to phagocytosis. Werb and Cohn (51) obtained evidence that the uptake of a certain area of plasma membrane around latex spheres was associated with an equivalent uptake of macrophage surface components like 5'-nucleotidase activity, cholesterol, and phospholipid. Hubbard and Cohn (30) radio-iodinated the surface of L cells and then compared the iodinated polypeptides of plasma membrane with that interiorized around latex phagolysosomes. Again the interiorized membrane was representative of the cell surface. In contrast, Tsan and Berlin (44) used purine and amino acid transport sites as plasma membrane markers and found that these sites were apparently excluded from the phagocytic vesicle. Nothing, however, is known about the makeup of pinocytic vesicle membrane. The HRP model does suggest at least two approaches to this problem. One is to try to separate pinocytic vesicles from plasma membrane by cell fractionation, using HRP as a specific biochemical and morphologic marker for the former. Another is to compare the rate at which defined membrane components enter the cell, relative to the rates of plasma membrane interiorization predicted by the stereologic analysis of HRP-reactive pinocytic vesicles.

The Secondary Lysosome Compartment

Previous studies indicated that all the HRP pinocytosed by macrophages and L cells entered the secondary lysosome compartment (40, 41). By cytochemistry, all cell-bound enzyme was contained within membrane-bound granules having a dense content of reaction product. More important, all cell-bound HRP was inactivated and degraded with first order kinetics, suggesting that it was being handled by a single degradative compartment. In this study, we found that cytochemically reactive HRP fills the entire secondary lysosome space. It begins to enter the compartment within 5 min of administration and gradually fills it up over another hour. Additional HRP exposure, though it results in further accumulation of HRP solute, does not expand the cytochemically reactive space. Also if HRP is given for 15 min and then the cells are chased for 45 min in enzyme-free medium, the dimensions of reactive lysosomes are similar to those of cells given HRP continuously for 1 h. We conclude that HRP can

be used to identify the entire existing lysosome compartment of cultivated cells.

The macrophages we studied had some 1,100 secondary lysosomes, most small in size. 92% had a diameter of less than $0.3 \mu\text{m}$ and thus could not be visualized in light microscope preparations. The lysosomes occupy some 2.5% of the cell volume and their surface area is 18% that of the cell. There may be another way of estimating the surface area of the lysosome space. Werb and Cohn (50) found that cultivated macrophages exchange radiolabeled cholesterol with serum lipoprotein carriers present in the culture medium. The cell-bound cholesterol is partitioned in two exchangeable pools: a rapidly exchanging compartment corresponding to the plasma membrane, and a more slowly exchanging one which includes secondary lysosomes and possibly pinocytotic vesicles as well. For cells cultivated in newborn calf serum, the rapid pool has twice the cholesterol as the slow. If the surface density of cholesterol in the two exchangeable compartments is similar, then the data indicate that the area of plasma membrane is about two times that of intracellular vacuoles. For our cells maintained in FCS, the stereologic approach showed that the cell surface area was three times the area of its vacuolar system. It is to be emphasized here that the vacuolar system is extremely sensitive to environmental conditions. Macrophages can greatly expand the lysosomal compartment by cultivation in newborn calf serum (7), by administration of nondigestible materials (e.g., 50), or by phagocytosis of particulates (e.g., 50). Relatively speaking, the cells cultivated in fetal calf serum exhibit a small lysosomal space.

The Fate of Pinocytosed Fluid

Our other unusual set of findings relates to the fate of pinocytotic vesicles in the cell. In essence, we noted that pinocytosis in macrophages brings in the equivalent of 26% of the cell volume and 190% of the cell surface area each hour, but that incoming vesicles then fuse with a secondary lysosome compartment which at all times represents just 2.5% of the cell volume and 18% of the cell surface area. Similar qualitative data were obtained with L cells though we did not establish that we had saturated the lysosome space. Thus, pinocytosis brings in as much as 10 times more fluid and membrane than can be accounted for in the secondary lysosome space.

We postulate that interiorized vesicle fluid must

rapidly egress from the vacuolar system, and then from the cell itself. This egress must be very rapid. We observed that incoming HRP fills the pinocytotic vesicle space within 5 min and that the latter does not expand further even though pinocytosis is preceding continuously. Therefore, after a short-life span, fluid must leave the pinocytotic vesicle compartment as fast as it is entering. The mechanism for this rapid efflux is unknown. We doubt that it is simply returned in a vesicular form to the extracellular environment. A priori this would make little sense, and we have quantitative and stereologic data that the initial rate of HRP uptake, during the first minutes before detectable lysosomal fusion, persists during the period in which incoming vesicles are fusing and apparently shrinking down in size. We also know that pinocytosed markers, like HRP (40, 41), inulin (Z. A. Cohn, unpublished observations), and colloidal gold (7) are not regurgitated into the culture medium once they are in the secondary lysosome pool; and there is no evidence that the acid hydrolases of lysosomes are exocytosed in large amounts under the culture conditions we utilized (e.g., 23).

More likely, interiorized fluid is lost by shifts across the newly forming secondary lysosome membrane and then from the cell itself. It is difficult to predict a mechanism for such shifts, but several observations should be kept in mind. Edelson and Cohn (14) noted that treatment of macrophages with con A resulted in the production of pinocytotic vesicles that could not fuse normally with secondary lysosomes. The cells accumulate large numbers of large pinocytotic vesicles, implying that lysosome fusion is required before incoming vesicles begin to shrink. Another fact is that the secondary lysosomes of cultivated mouse macrophages (8, 17) and human fibroblasts (38) are permeable to a variety of small hydrophilic solutes, e.g., amino acids and saccharides, but are impermeable to larger solutes. The vacuolar space swells dramatically if cells interiorize large solutes, e.g., oligosaccharides and peptides, for which they lack degradative enzymes. This could mean that fluid moves into the vacuole because of a high intravacuolar osmotic pressure, or that a normal efflux of fluid towards a high cytoplasmic osmotic pressure is counterbalanced by the presence of indigestible solute. In any case, the vacuolar membrane behaves as a semipermeable one. Fluid shifts across a semipermeable membrane may be initi-

ated by many factors. Conceivably, the lysosome delivers macromolecular ions into the pinocytic vesicle which form complexes capable of reducing the osmotic pressure of the newly formed secondary lysosome, a mechanism which has been proposed for the condensation of secretory vacuoles (42). Or the incoming vesicle membrane may be considerably altered after fusion with lysosomes. The studies of Henning and associates (25, 26, 32), have pointed to differences in the lipid, polypeptide, and enzymatic composition between membranes of liver secondary lysosomes (loaded with Triton) and plasma membrane. In addition, the lysosome contents such as acid and enzymes may be capable of altering the composition or organization of the incoming vesicle membrane; e.g., Hubbard and Cohn demonstrated that iodinated plasma membrane polypeptides were rapidly degraded when trapped in a latex phagolysosome (30). One plasma membrane activity that may be relevant here is the ability to actively exclude certain salts from the cytoplasm. If this is inactivated after secondary lysosome formation, a salt flux from vacuole into cytoplasm may ensue. By analogy with transepithelial salt and water systems (10, 12), such differences in the secondary lysosome membrane relative to the plasma membrane may allow for the bulk movement of salt and water through the cytoplasm and out of the cell, even though the pinocytosed droplet of fluid and the culture medium are initially identical in solute composition.

The Fate of Interiorized Membrane

The fate of the large amounts of interiorized vesicle membrane after fusion with lysosomes is also unclear. We doubt, however, that it is significantly degraded within the lysosome, for this would lead to a high rate of plasma membrane turnover, i.e., twice the macrophage surface and one-half the L cell surface each hour. What little is known about membrane turnover in cultivated cells suggests that components are replaced at very slow rates. Warren and Glick noted that reutilizable amino acid and sugar precursors incorporated into the plasma membrane of growing L cells turned over at negligible rates (48). Hubbard and Cohn (30) found that L cell plasma membrane proteins, tagged with a nonreutilizable radioiodine label, were degraded to amino acids at a rate of only 2% per hour., although these labeled components were degraded much more rapidly when

interiorized membrane was trapped around an indigestible latex particle. Devreotes and Fambrough (11) likewise noted that the acetylcholine receptor of developing muscle cells in vitro turned over with a t one-half of some 22 h. Although none of these studies established that membrane turnover occurs via pinocytosis, the existing data do state that turnover by any means is much slower than if all pinocytosed membrane were degraded within lysosomes. It is still possible that some components of the interiorized membrane, not tagged by the above labels, are degraded at rapid rates as a result of pinocytosis.

If interiorized membrane were degraded by lysosomes, it would also follow that cells must be capable of resynthesizing membrane components at rapid rates. Macrophages do not appear to be capable of such rapid synthesis. For example, trapping of large amounts of plasma membrane in latex phagolysosomes results in the interiorization of surface 5'-nucleotidase activity, cholesterol, and phospholipid (51). These components are replaced by processes involving new protein synthesis, but only after a 6 h lag period. Although we know little about the rates and mechanisms for cell membrane formation, the suggestion at this time is that cells could not immediately replenish the large amounts of membrane that would be lost if pinocytosed membranes were completely degraded in lysosomes.

Recycling of Pinocytosed Membrane

The most likely explanation of our data on the uptake and fate of incoming pinocytic vesicle membrane is that it is recycled back to the cell surface after shrinkage of the newly formed secondary lysosome. During such recycling, non-permeable solutes like HRP, and acid hydrolases themselves probably are not regurgitated into the extracellular space nor leaked into the cytoplasm in significant amounts. A plausible morphologic mechanism for membrane recycling is that it involves the production of tiny vesicles capable of returning large amounts of surface membrane, with very little content, to the cell surface. Such tiny vesicles are abundant in the Golgi region, where many of the events being discussed, i.e., secondary lysosome formation and shrinkage, are taking place. Sometimes large numbers of tiny vesicles are found in association with larger presumptive pinocytic vesicles (29). Previously these images were assumed to represent vesicles about to

fuse with the pinocytotic vesicle rather than vesicles that were being pinched off from it. Perhaps coated vesicles are specialized for cell membrane assembly, as has previously been suggested (21, 39). These possibilities are quite speculative, and it is possible that membrane recycling proceeds by a nonvesicular mechanism.

The idea of membrane recycling via pinocytosis has occurred in many studies of secretion of membrane-bound materials. For example, in neurohypophysis (13), anterior pituitary (20), adrenal medulla (1), peripheral nerve terminals (5, 27), and islet cells (36), it has been suggested that membrane components delivered to the cell surface during the exocytosis of the secretory granule are retrieved by the cell through a burst of pinocytotic activity. This hypothesis has not been tested quantitatively, but a stereologic analysis with HRP might be a useful means of doing so. L cells have long been employed as models for the study of connective tissue matrix secretion, and it has recently become apparent that macrophages are capable of secretory activity as well (23, 45). Identification of the presumptive secretory droplets in these cells is not yet possible in routine sections so that we cannot verify whether the surface area associated with the secretory process is similar to that involved in pinocytotic vesicle formation.

Our current studies are directed to finding other quantitative approaches for verifying the main implications of this study, viz, that during pinocytosis, a substantial proportion of the cell surface flows into the cell, through the vacuolar system, and then back to the cell surface.

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