

# PINOCYTOSIS IN FIBROBLASTS

## Quantitative Studies In Vitro

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### ABSTRACT

Horseradish peroxidase (HRP) was used as a marker to determine the rate of ongoing pinocytosis in several fibroblast cell lines. The enzyme was interiorized in the fluid phase without evidence of adsorption to the cell surface. Cytochemical reaction product was not found on the cell surface and was visualized only within intracellular vesicles and granules. Uptake was directly proportional to the administered concentration of HRP and to the duration of exposure. The rate of HRP uptake was 0.0032–0.0035% of the administered load per  $10^6$  cells per hour for all cells studied with one exception: L cells, after reaching confluence, progressively increased their pinocytic activity two- to fourfold. After uptake of HRP, L cells inactivated HRP with a half-life of 6–8 h. Certain metabolic requirements of pinocytosis were then studied in detail in L cells. Raising the environmental temperature increased pinocytosis over a range of 2–38°C. The  $Q_{10}$  was 2.7 and the activation energy, 17.6 kcal/mol. Studies on the levels of cellular ATP in the presence of various metabolic inhibitors (fluoride, 2-desoxyglycose, azide, and cyanide) showed that L cells synthesized ATP by both glycolytic and respiratory pathways. A combination of a glycolytic and a respiratory inhibitor was needed to depress cellular ATP levels as well as pinocytic activity to 10–20% of control values, whereas drugs administered individually had only partial effects. In spite of the availability of an accurate quantitative assay for fluid and solute uptake, the function of pinocytosis in tissue culture cells remains unknown.

Pinocytosis, the interiorization of droplets of fluid from the environment into cells, was described in mammalian cells in vitro by Lewis long ago (34, 35). Most of the work has since focused on mononuclear phagocytes (macrophages), possibly because they exhibit such active pinocytic activity. Relatively little is known about this phenomenon in other cultivated cells, e.g., for many cells the functional significance and metabolic parameters of pinocytosis are unclear. In a previous study (53) on pinocytosis in macrophages, the usefulness of horseradish peroxidase (HRP) as a marker solute

for quantitative work was demonstrated. Its two main advantages were that the amount of cell-bound HRP could be quantitated by a sensitive (nanograms per milliliter) enzymatic assay, and its distribution in cells could be discretely localized by the cytochemical technique of Graham and Karnovsky (24). In this paper, the HRP model is extended to the study of mouse fibroblasts in vitro. We have obtained quantitative data on several variables in pinocytosis including the effects of temperature, cell growth characteristics, and metabolic inhibitors.

## MATERIALS AND METHODS

### Reagents

Fetal calf serum (FCS), newborn calf serum, minimal essential medium (MEM), Dulbecco's minimal essential medium, HEPES buffer were obtained from the Grand Island Biological Co., Grand Island, N. Y. Type II and Type III 2-deoxyglucose (2-DG), adenosine triphosphate (ATP), desiccated firefly lantern extract, type II horseradish peroxidase (HRP), diaminobenzidine (DAB), *o*-dianisidine, and Triton X-100 were obtained from the Sigma Chemical Corp., St. Louis, Mo. Sodium cyanide (NaCN), sodium azide (NaN<sub>3</sub>), and sodium fluoride (NaF) were analytical grade reagents from Matheson Coleman & Bell, Norwood, Ohio. Trypsin and hen egg white lysozyme, twice crystallized, came from the Worthington Biochemical Corp., Freehold, N. J. Bovine serum albumin, Fx V (BSA), human serum albumin (HSA), rabbit gamma globulin, Fx II (RGG) were obtained from Miles Laboratories, Inc., Kankakee, Ill. Carrier-free sodium<sup>125</sup> iodide, 20 mCi/ml came from New England Nuclear, Boston, Mass.

### Cells

Three continuous fibroblast lines were maintained as cell monolayers on Falcon (Baltimore Biological Laboratories, Baltimore, Md.) plastic petri dishes (35, 60, and 100 mm in diameter). L cells were provided by Drs. S. Silverstein and A. Hubbard and were maintained in 5% FCS-MEM supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). 3T3 and SV40-transformed 3T3 cells were given to us by Dr. L. Ossowski and were cultivated in 10% FCS-Dulbecco's MEM supplemented with antibiotics. The 3T3 cells were originally considered to be a fibroblast derivative (55), though some suggest now that it is of endothelial origin (42). Calf embryo fibroblasts were provided by Dr. N. M. y Lopez. Primary cultures were made from embryonic thigh muscles in 10% FCS-Dulbecco's MEM with antibiotics. After three passages the cultures consisted entirely of fibroblastic vs. muscular elements. All monolayers were subcultivated after removal from the petri dishes with purified trypsin (100 µg/ml phosphate-buffered saline).

### Quantitative Assays for Endocytosis

Crystalline, highly soluble HRP was administered to cell monolayers, generally for 1 h at a final concentration of 1 mg/ml of culture medium. The amount of HRP pinocytosed in this interval is minute, relative to the concentration of enzyme in the culture medium (v.i.), so that care must be taken to remove noncell-bound HRP from all parts of the culture vessel. Of particular importance is that HRP adheres to the surface of the culture dish itself, and this compartment elutes only slowly during the washing procedure (53). Accordingly, cells were

washed five times over a 15–20-min period in serum-free culture medium and were then returned to the 37°C incubator for 30 min in the presence of 5% FCS-MEM; this step allowed remaining dish-bound enzyme to be eluted. The monolayers were rinsed two more times in phosphate-buffered saline and lysed in 0.05% (vol/vol) Triton X-100 in water. The total time from removal of medium containing HRP to lysis of the cells was thus about 1 h, during which interval cells inactivated less than 10% of the total cell-bound enzyme (v.i.). Aliquots of the cell lysate were assayed for bound HRP by an enzymatic assay, which has hydrogen peroxide and *o*-dianisidine as substrates, and detects as little as 1 ng/ml of HRP (53). The rate of production of oxidized *o*-dianisidine (absorbing at 460 nm) was directly related to the concentration of enzyme present (Fig. 1). All cells used had negligible amounts of endogenous peroxidatic activity. Identical standard curves relating enzyme concentration to rate of increase in absorbance 460 nm were obtained when standards were assayed in the presence or absence of cell lysates.

The enzymatic assay thus measures the amount of HRP by weight interiorized, which can be expressed on a per cell, per culture, or per unit of cell protein basis. Cell protein was measured on the Triton lysates using a modification of the Lowry method with hen egg white lysozyme as standard (36). The standard deviation of cell protein measurements in groups of eight replicate cultures was 8%.

Endocytosis of soluble HRP was also quantitated in suspension cultures of L cells maintained on a roller

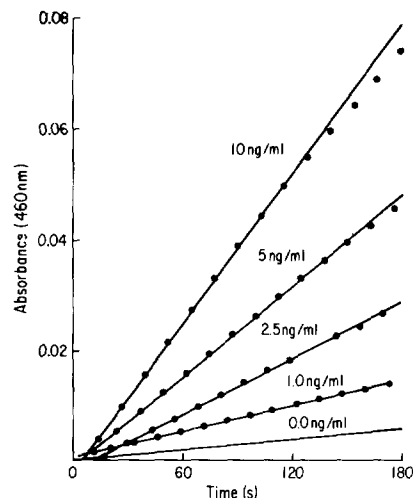


FIGURE 1 Detection of HRP by enzymatic assay. The kinetics of oxidation of *o*-dianisidine ( $10^{-4}$  mM) in the presence of  $H_2O_2$  ( $10^{-3}$  mM) and varying concentrations of HRP, Sigma type II, is followed at 460 nm in 0.1 M phosphate buffer, pH 5.0. The reaction proceeds linearly for 1–3 min, and its rate is directly related to the concentration of enzyme present.

device. After exposure to enzyme, the cells were centrifuged at 80 *g* at 4°C for 10 min, resuspended in wash medium, transferred to a fresh tube, washed four more times so that the last wash was free of enzymatic activity, and lysed in detergent.

Radiolabeled proteins—BSA, HSA, RGG, and lysozyme—were also used as soluble protein markers for pinocytosis. 20 mg of each were radioiodinated with 1–2 mCi carrier-free [<sup>125</sup>I]Na, in 1 ml of 0.2 M sodium phosphate buffer pH 7.4, using hydrogen peroxide (5 μl of a 0.030% solution every 5 min three times) and 25 μl of an insolubilized lactoperoxidase preparation (coupled to Sepharose 2B with cyanogen bromide and kindly provided by Dr. S. Silverstein). After iodination, all preparations were dialyzed against normal saline to remove free <sup>125</sup>I. Specific activities of 0.01–0.10 μCi/μg protein were obtained. Cell monolayers were exposed to the marker proteins at 1 mg/ml in culture medium, washed and lysed as described for HRP, and assayed for total and trichloroacetic acid-precipitable counts in a Packard gamma scintillation counter, model 5220 (Packard Instrument Co., Inc., Downers Grove, Ill.).

Preparation and uptake studies on HRP-antiHRP immune aggregates were performed as previously described (54). Exposure was always in the presence of 5% FCS, since complexes in the presence of fibroblasts and absence of serum adhered firmly to the culture vessel itself.

### *Inactivation of HRP by L Cells*

Studies on the fate of HRP pinocytosed by L cells were carried out in a fashion similar to that previously employed in macrophages (53). Cultures were allowed to pinocytose HRP, washed, and then followed for an appropriate length of time in HRP-free culture medium. At each time point, duplicate cultures were assayed for enzyme remaining in cells, as well as enzyme in the culture medium; this was an attempt to detect enzyme being exocytosed from the fibroblasts. The HRP system is suitable for detecting small amounts of exocytosed enzyme, if such a process occurs. Small concentrations (nanograms per milliliter) are stable in the presence of serum proteins at 37°C for several days, even in the presence of cells, since the rate of pinocytosis of soluble enzyme is so slow.

### *Effects of Temperature on Endocytosis*

The effect of temperature on the rate of pinocytosis of HRP was most easily examined in cultures buffered with 25 mM HEPES rather than bicarbonate-CO<sub>2</sub>. This buffer did not alter the rate of pinocytosis. The culture vessels were surrounded by a few millimeters of water on a stage, itself supported in a large reservoir. The water temperature could then be kept below ambient temperature by adding ice chips, or above, by a suitable water bath. Cells were equilibrated for 30 min before the ad-

ministration of HRP. Data obtained at 4°, 19°, 30°, and 37°C with bicarbonate-buffered media were identical to those obtained with the HEPES system. In order to obtain measurable uptake of HRP at low temperatures, we used a 1-h exposure of 2 mg/ml HRP and monolayers which had just reached confluence on 60-mm dishes.

### *Metabolic Inhibitor Studies*

All inhibitors were administered to L cells in fresh culture medium for a total of 2 h; 1 h before and 1 h during the addition of HRP. The 2-h treatment produced no decrease in cell number or total protein, and cell growth resumed normally after removal of the drugs. Cyanide, azide, and fluoride decreased the enzymatic activity of HRP 50% in the concentrations administered to cells. However, this decrease in activity was entirely reversible with a 10-fold dilution. Since the volume of cells was at least 1/1,000th the final volume of the cell lysate assayed, the presence of inhibitor would not influence the enzymatic assay.

### *Cellular ATP Determinations*

Total L-cell ATP levels were determined by a luciferin-luciferase assay, similar to the system of Stanley and Williams (52), in which the production of light is related to the amount of ATP present. Luminescence was detected with a Mark II Nuclear Chicago scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.), set for out-of-coincidence counting, 100% amplification, and window limits of six to seven. Samples were counted for two 6-s intervals and the second count was used, since vial luminescence was lower. Standard ATP solutions (10<sup>-9</sup>–10<sup>-11</sup> mol/ml) were prepared from a 10<sup>-6</sup> mol/ml frozen stock solution. For cellular ATP assays, monolayers were rinsed in PBS, placed in 2 ml cold distilled water, scraped from the dish, frozen in dry ice acetone, boiled 5 min, and centrifuged for 10 min at 160 *g* at 4°C, and the supernate (25–50 μl) was assayed. The levels of ATP/10<sup>6</sup> cells varied from 4.5 nmol for sparse cultures (200 μg protein/60-mm dish) to 1.7 nmol for overgrown confluent cells (1,200 μg protein/60-mm dish). The presence of metabolic inhibitors did not influence the assay for ATP.

### *Cytochemistry*

Monolayers which had been exposed to HRP were generally washed quickly (two to three times in saline over 1–2 min), and then fixed in 2.5% glutaraldehyde buffer with 0.1 M Na cacodylate buffer, pH 7.4 at room temperature for 5 min. The cells were washed three times, and cell-bound HRP was localized with the diaminobenzidine procedure of Graham and Karnovsky (24), using a 10-min exposure to 50 mg% DAB in 0.05 M Tris buffer, pH 7.6 and 0.01% hydrogen peroxide. For electron microscopy, cells were postfixed in 1% osmium tetroxide in 0.1 M Na cacodylate, pH 7.4, followed by

0.5% magnesium uranyl acetate in normal saline, pH 5.0, each for 1 h at 4°C. Cells were dehydrated in graded alcohols and embedded in Epon. The monolayers were processed as cell pellets according to Hirsch and Fedorko (28), or as monolayers for oriented sections according to Ross (45).

## RESULTS

### *HRP as a Marker Solute for the Study of Pinocytosis in L Cells*

**CYTOCHEMISTRY:** Previous studies with a variety of endocytic markers in many cell types (e.g., 17, 22) have demonstrated that these materials are interiorized into membrane-bound vesicles. After one or more fusions with acid hydrolase-containing granules, secondary lysosomes are formed in which the endocytosed materials are sequestered and/or digested, usually in the Golgi region. That HRP is interiorized by L cells by a similar endocytic process was demonstrated cytochemically in cells exposed to 1 mg/ml of enzyme. After brief (5–10 min) exposures to HRP, the enzyme was localized in small vesicles of varying size, often near the cell surface (Fig. 2). These newly arising pinocytic vesicles always contained just a peripheral rim of reaction product. If longer (60-min) HRP exposures were used, reaction was found throughout the vesicles and granules that constitute the vacuolar apparatus. Most HRP-containing structures were replete with reaction product but some exhibited a peripheral rim of reactivity characteristic of newly arising vesicles (Fig. 3). If cells exposed for 60 min to HRP were washed and incubated for 0.5 h before fixation, reaction product was restricted to dense granules localizing in the perinuclear or Golgi region (Fig. 4). Presumptive pinocytic vesicles lacked HRP. These observations suggest that HRP is taken up in vesicles which, after fusion with each other and with pre-existing lysosomes, accumulate as granules in the Golgi zone. These secondary lysosomes probably contain a higher concentration of enzyme than that originally pinocytosed. Enzyme was never found in other cell organelles (Golgi apparatus, rough endoplasmic reticulum, mitochondria) or bound to the cell surface (Figs. 2, 3, 4).

**QUANTITATION OF HRP UPTAKE:** Quantitation of uptake by the enzymatic assay established that HRP is continuously taken into cells as a solute in pinocytic droplets, without itself altering the rate of ongoing pinocytosis. In both sparse and

confluent L-cell cultures, uptake increased linearly with the concentration of HRP in the medium over a large range (100–2,000  $\mu\text{g/ml}$ ) (Fig. 5 A). This suggests that HRP is imbibed as a solute in pinocytic droplets without prior binding to the cell surface. If surface binding had to precede endocytosis (adsorptive vs. fluid endocytosis [30]), then one would expect some saturation in the uptake rate with increasing concentrations of enzyme in the environment. At any given concentration of HRP in the culture medium, the uptake of enzyme proceeded at a constant rate over a several hours period of exposure, and the plot of uptake vs. exposure time (Fig. 5 B) passed through the origin. This means that there was no adsorption phase preceding uptake. The fact that uptake was linearly related to HRP concentration and exposure time suggests that HRP is not itself altering ongoing pinocytic activity in L-cells.

To further verify that HRP does not itself influence the rate of ongoing endocytosis in L-cells, we measured the rate at which other soluble proteins, radiolabeled with  $^{125}\text{I}$ , were bound to monolayers in the presence or absence of 1 mg/ml HRP. Using a protein load and a washing procedure similar to that employed for HRP, we found that the binding of BSA, HSA, RGG, and lysozyme was not altered by addition of HRP to the medium, and that these proteins did not alter HRP uptake (Table 1). Of concern, however, was that the apparent rates of uptake of these other solutes differed considerably from one another and from HRP, and even varied from one experiment to another. Control studies showed that all four radiolabeled proteins exhibited variable but significant adsorption to the culture vessel and/or the cells. This was demonstrated directly by exposing dishes without cells to the four markers, and indirectly by kinetic studies in which much of the binding to cell monolayers (e.g., 80–90%) occurred within 15–30 min, with little increase thereafter.

The rate of endocytosis of HRP by L-cells can therefore be expressed with some degree of precision. In terms of the percent of the administered concentration of HRP in the medium, 0.0032–0.0035% was interiorized by  $10^6$  growing cells per hour. The value was of the order of 0.1 ng/ $\mu\text{g}$  cell protein/h when expressed per unit of cell protein at a load of 1 mg/ml. Although the percent of the administered load interiorized was tiny, we could obtain no evidence that this cell-bound material represented a susceptible subfrac-

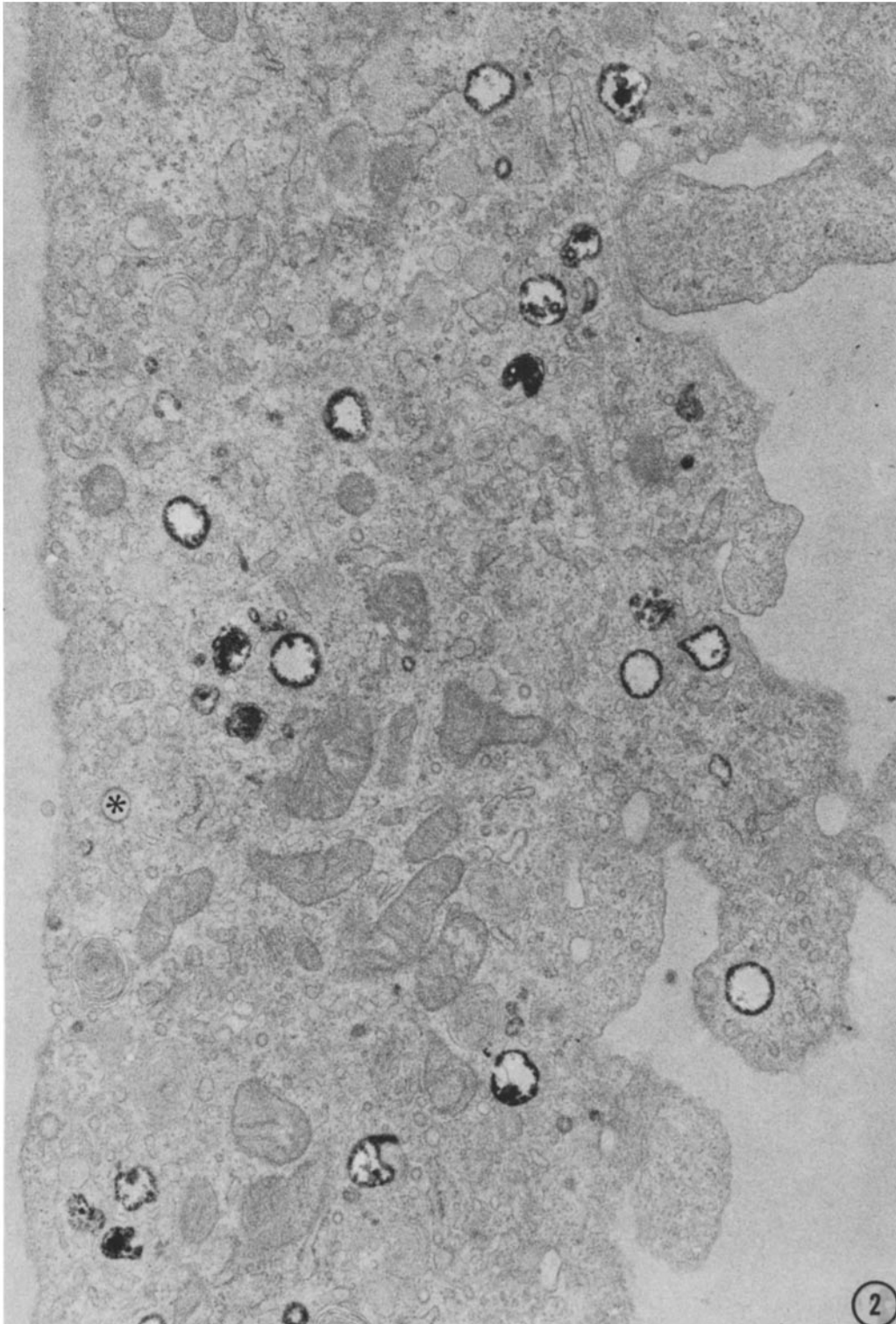
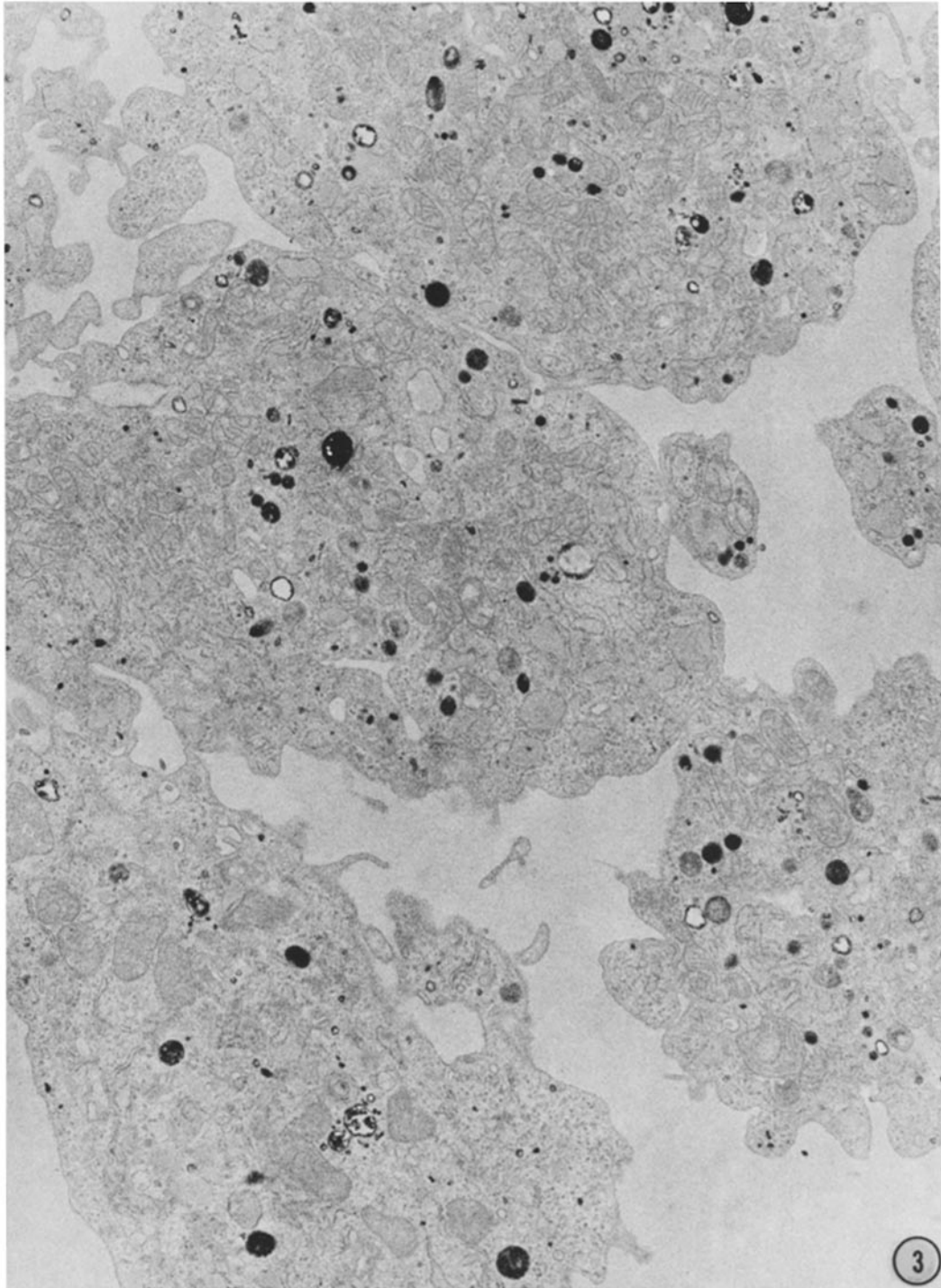


FIGURE 2 Pinocytotic vesicles in L cells. L cells were exposed for 10 min to 1 mg/ml HRP, washed quickly, fixed, and processed to localize cell-bound enzyme with the diaminobenzidine- $\text{H}_2\text{O}_2$  substrate mixture. The sections were not stained with lead or uranyl salts. Reaction product is found in vesicles of varying size, often close to the cell surface. HRP cannot be detected on the cell surface itself, or in coated vesicles (\*), mitochondria, and rough endoplasmic reticulum.  $\times 23,000$ .



**FIGURE 3** Distribution of HRP in the vacuolar apparatus. L cells were exposed for 60 min to 1 mg/ml HRP and washed quickly before processing. The section was not stained with heavy metals. Reaction product fills granules of varying size, and occupies the periphery of some vesicles, similar to those seen in cells exposed only 10 min to enzyme. Presumably, the concentration of HRP in incoming pinosomes is increased after fusions with lysosomes and shrinkage of their content.  $\times 8,500$ .

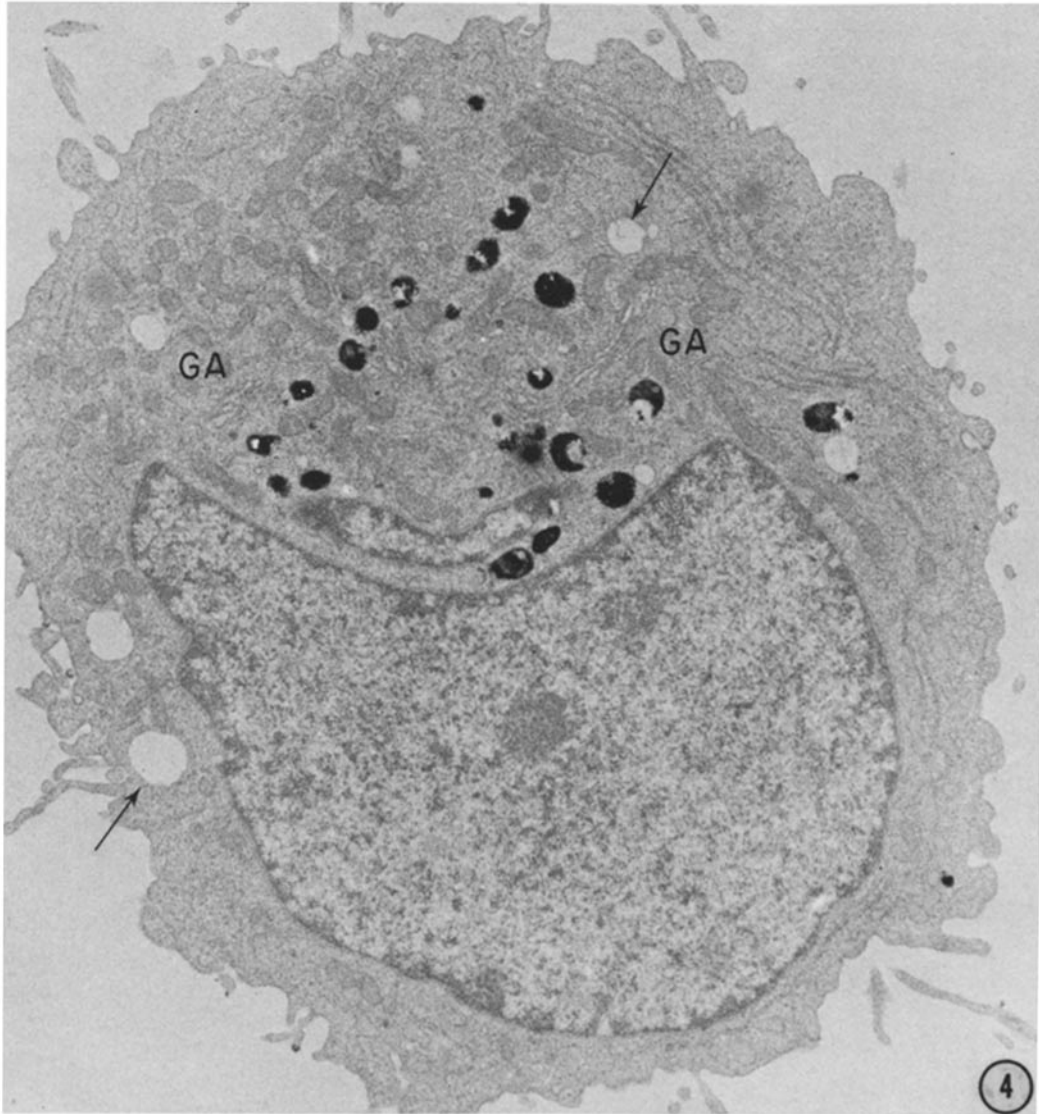


FIGURE 4 Accumulation of HRP in the Golgi region. L cells were exposed for 60 min to 1 mg/ml HRP, washed, and maintained in HRP-free culture medium for 30 min before fixation and processing. The section was unstained with heavy metals. Reaction product is now found only in dense membrane-bound granules near the Golgi apparatus (GA). Presumptive incoming pinocytotic vesicles (arrows) are negative.  $\times 9,500$ .

tion of the total administered. Successive transfers of medium containing HRP from one monolayer to another yielded identical uptake rates for all six such transfers attempted.

**FATE OF HRP:** In order for a soluble protein to be useful as a marker for pinocytosis, it must be digested relatively slowly by the cell. A prolonged

(1-h) washing procedure is required to remove HRP which is bound to the culture vessel (53). The relatively slow inactivation of HRP was demonstrated directly by following the fate of enzyme interiorized by both growing and confluent L-cells (Fig. 6). The amount of cell-bound enzyme diminished exponentially with time until it was no longer

detectable. The half-life was 6–8 h in seven experiments, corresponding to an inactivation rate of 8–10% per hour. This disappearance rate is identical to that observed for HRP pinocytosed by

mouse macrophages (53). Also, as in the macrophage studies, exocytosis of even small amounts of enzyme (2 ng or more) from L-cells into the culture medium was not detected.

### Some Variables in the Activity of Fibroblast Pinocytosis in Vitro

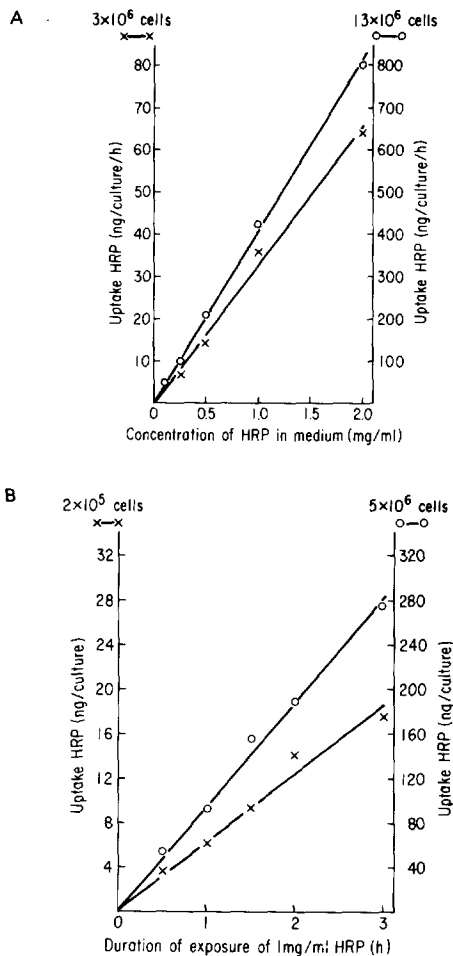
**CULTURE MEDIUM:** The uptake of HRP was similar in the presence of varying concentrations (1–40%) of fetal and newborn calf serum. Previous studies had demonstrated that the rate of pinocytic vesicle formation in macrophages was greatly dependent on the concentration of newborn calf serum in the culture medium (14). However, none of the sera currently available to us stimulated the formation of phase-lucent vesicles, either in L cells or in macrophages.

Some properties of cells in vitro can be altered by the addition of fresh culture medium or fresh serum to replace medium previously conditioned by the presence of cells. In quantitating endocytic rates, it was simplest to add HRP to the cells in a known volume of fresh medium. Use of medium conditioned 1–4 days by L cells did not consistently, nor significantly (more than 10–15% decrease) alter HRP uptake. Also the rate of pinocytosis was unchanged when the frequency at which culture medium was changed before assay was varied from 1-, 2-, 3-, or 4-day intervals.

**SUSPENSION CULTURES:** The uptake of HRP by suspension cultures of L cells was similar to that by monolayers. This applied to cells either maintained continuously in suspension or freshly placed in suspension after trypsinization of monolayer cultures. However, performance of pinocytosis assays on suspension cultures was technically more difficult, in that multiple cell centrifugations and resuspensions were required.

**CELL CYCLE:** At both light and electron microscope levels, it appeared that the uptake of HRP into membrane-bound granules occurred to a similar extent in all cells in the culture, even cells in mitosis. Since the cells were heterogeneous with respect to cell cycle phase, this observation suggests that pinocytic activity persists throughout most, if not all, of the cycle. However, the cytochemical technique does not permit one to say that individual cells are quantitatively similar, since the amounts of enzyme within visualized granules is conceivably quite different.

**DENSITY OF CELLS ON THE MONOLAYER:** The rate of pinocytosis was examined in sparse



**FIGURE 5** Kinetics of HRP uptake. The uptake of HRP into preconfluent (x—x) and confluent (O—O) L-cell monolayers was quantitated using an enzymatic assay. (A) During a 1-h incubation, the amount of HRP bound to L cells is linearly related to the concentration of enzyme in the culture medium, as would be expected of a material being endocytosed as a solute in pinocytic droplets. Uptake shows no evidence of saturation even at high concentrations of enzyme (2 mg/ml). (B) At any given concentration of HRP in the medium, in this case 1 mg/ml, the uptake of HRP increases linearly with the time of exposure to enzyme, indicating that pinocytosis of this marker proceeds continuously at a constant rate. The linear relation passes through the origin, showing that binding of HRP to the culture dish or to the cell surface is undetectable.



TABLE I  
Influence of HRP on the Uptake of Other Soluble Proteins

Protein	Uptake of radiolabeled protein		
	In absence of HRP	In presence of HRP	Uptake of HRP
	ng	ng	ng
Bovine serum albumin (BSA)	30 ± 6	27 ± 8	32 ± 1.5
Human serum albumin (HSA)	71 ± 9	70 ± 4	33 ± 1.5
Rabbit gamma globulin (RGG)	135 ± 11	146 ± 15	32 ± 1.2
Hen egg white lysozyme	508 ± 18	465 ± 54	32 ± 1.0

L cells were exposed to four different radiolabeled proteins in the presence or absence of 1 mg/ml HRP. After a 1-h exposure to 1 mg/ml of protein, the monolayers were washed, as described in Materials and Methods, and then lysed to determine the amount of bound radiolabel. The data are from a single set of replicate cultures using triplicates for each point. All proteins were bound to a similar extent in the presence or absence of HRP, and none altered the level of HRP uptake. The proteins all seem to be interiorized at different rates, but much of the bound label probably represents material absorbed to the cells and/or the culture vessel.

and confluent (i.e., the entire dish surface is covered by cells) monolayers. A true stationary phase does not exist in L cells in that the number of cells, as well as the total protein in the monolayer, continues to increase slowly after confluence is reached (Fig. 7 A). Confluent cells were found to be more active in their pinocytotic activity. Two approaches were used with similar results. In one, cells were harvested from stock cultures, plated in different numbers ( $2-16 \times 10^5/60\text{-mm}$  petri dish), and tested 2 days later. In the other, cells were plated initially in low numbers ( $2 \times 10^5/\text{dish}$ ) and then the monolayers were followed at frequent intervals, with culture medium changes every 2-3 days.

During the rapid-growth phase, L cells pinocytosed at a constant rate, i.e., 32-35 ng/ $10^6$  cells/h at an HRP load of 1 mg/ml (Fig. 7 B). Once confluence was reached, the uptake both per cell and per unit of cell protein began to increase progressively and substantially (two- to fourfold) with time (Fig. 7 B). This increase could not be altered by increasing the frequency at which the culture medium was changed. When medium from confluent cultures was added to growing cells, their pinocytotic activity was not altered. The enhanced rate of uptake of HRP in confluent cultures remained concentration- and time-dependent, as in growing cells (Fig. 5 A, B).

The morphology of cells in confluence differed from cells in growth phase. The latter were flattened along the surface of the culture vessel and had many microvilli projecting into the culture medium. Confluent cells covered a much smaller area of the dish surface, and most of the basal

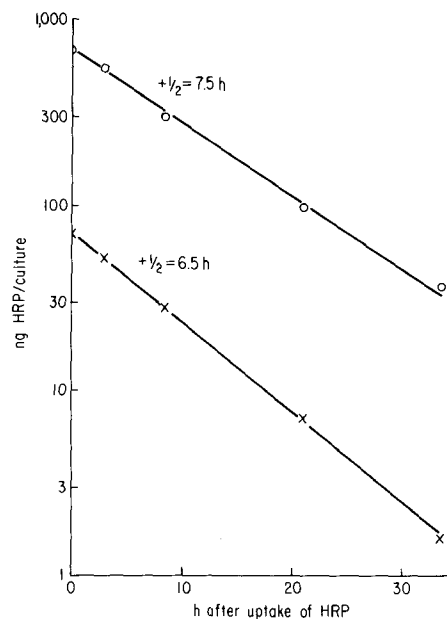


FIGURE 6 Fate of HRP pinocytosed by L cells. After a 1-h exposure to enzyme, pre-confluent (x-x) and confluent (o-o) monolayers were washed and incubated in HRP-free culture medium. The amount of HRP per culture diminishes exponentially until enzyme cannot be detected. The half-lives varied from 6 to 8 h in several experiments. Enzyme is probably being inactivated by lysosomal hydrolases. Exocytosis of cell-bound HRP into the culture medium is not detectable, and the cells continue to grow, firmly adherent to the monolayer.

portion was not closely apposed to the dish. The cell surface facing the culture medium was extremely irregular with many extensions (villi, blebs, ruffles) of varying size and shape (Fig. 8 A).

When exposed to HRP, even for brief intervals, confluent cells contained typical HRP-reactive vesicles and in addition some very unusual, HRP-positive, membrane-bound structures (Fig. 8 B, arrows). These "pinosomes" had extended shapes with many cytoplasmic protrusions into the center.

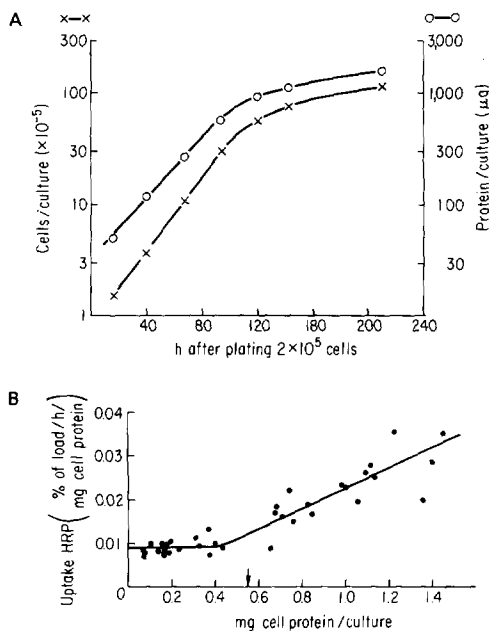


FIGURE 7 (A) Growth curves of L-cell monolayers. Cell protein, or more correctly, monolayer protein (O—O), and cell number (x—x) increase exponentially after the plating of  $2 \times 10^5$  cells on 60-mm plastic petri dishes in 5% FCS-MEM. The doubling times are 22 h and 18 h, respectively. After confluence is reached (110 h), the cells continue to grow, but at a much reduced rate. (B) Variation of HRP uptake with monolayer cell density (protein). The rate of pinocytosis does not change significantly in rapidly growing cultures, but after confluence is reached (arrow), it increases progressively several fold. The data for confluent cells fit a straight line with a correlation coefficient of 0.83.

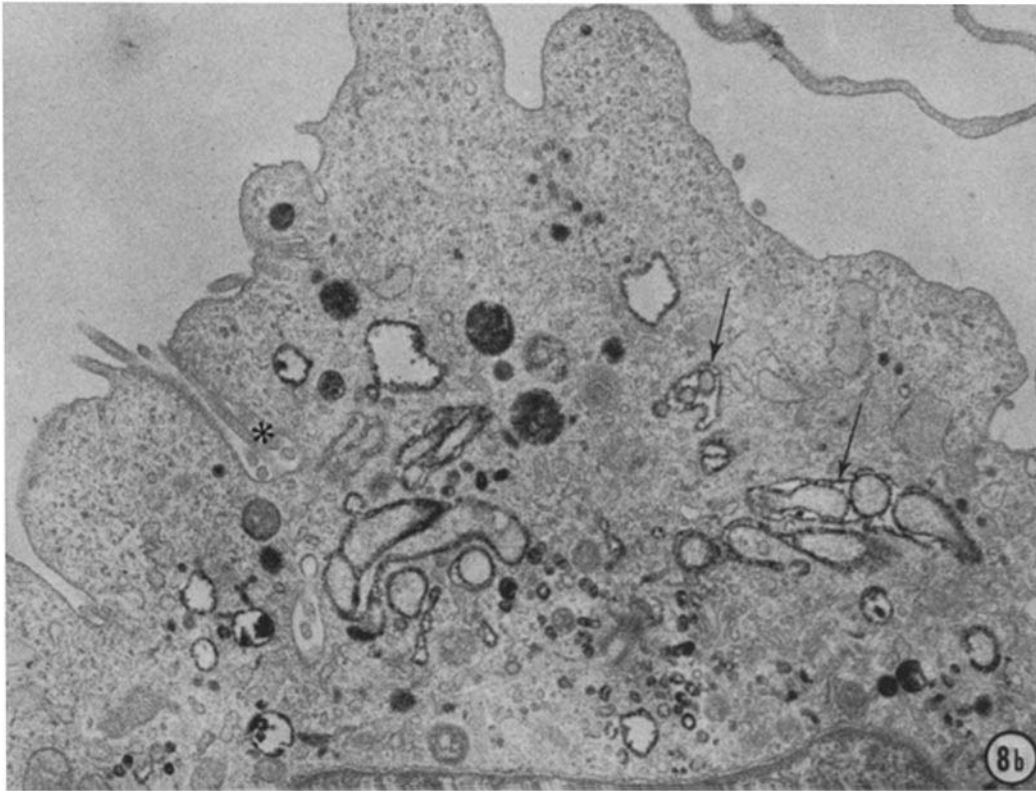
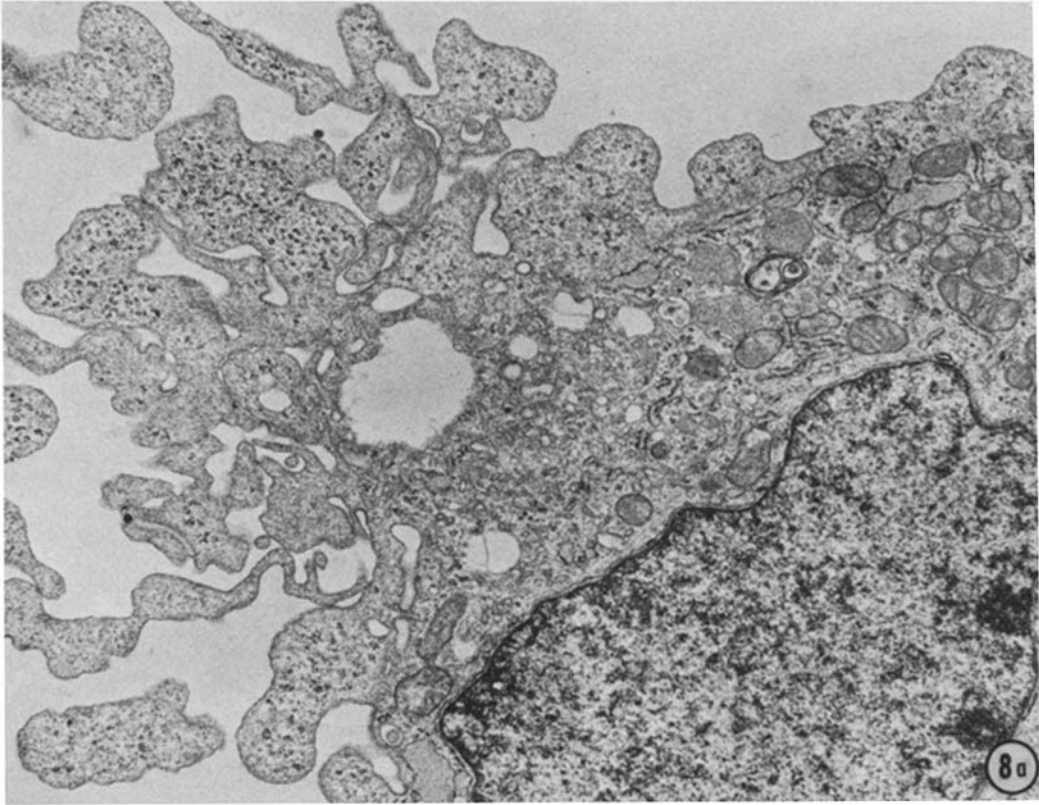
Serial sectioning showed that they were entirely intracellular. This would be expected from their content of HRP, since soluble enzyme should be washed out if an incoming endocytic structure had not completely pinched off from the cell surface. We suspect that these unusual endocytic "vesicles" can arise in two ways: by fusion of the tips of highly irregular cell surface projections, (e.g., Fig. 8 A); or by pinching off invaginated channels (Fig. 8 B).

The increased pinocytic activity of confluent monolayers was reversible, but only slowly. Confluent cultures were trypsinized and replated in sparse ( $3 \times 10^5$  cells/60-mm dish) numbers, and the cells were then exposed to HRP for 1 h at varying time periods after replating. Enhanced uptake of HRP appeared to persist unchanged for some 10 h even though the monolayers were not confluent and had resumed growth (Table II). Only by 24–32 h did the pinocytic activity return to normal. The increased pinocytic activity of replated confluent cells was similar, regardless of the density (subconfluent, confluent) at which they were replated. Conversely, an increase in pinocytic activity was immediately observed when rapidly growing cells were trypsinized and replated at high densities (800–1,000  $\mu\text{g}$  protein/dish).

**OTHER CELL TYPES:** Other sources of fibroblasts pinocytosed at a rate identical to that observed for growing L cells. In nanograms HRP interiorized per micrograms cell protein per hour at a load of 1 mg/ml HRP, the rates were  $0.109 \pm 0.018$ ,  $0.105 \pm 0.013$ , and  $0.103 \pm 0.009$  for 3T3, SV40-transformed 3T3, and calf embryo fibroblasts, respectively. Both the untransformed 3T3 and the calf embryo cells could be maintained in a nongrowing confluent condition for several days, but in these instances, no increase in pinocytic activity was observed.

**ANTIBODY:** Previous studies on mouse macrophages (54) demonstrated that the formation of

FIGURE 8 Electron micrographs of confluent L cells. (A) A cell sectioned perpendicularly to the surface of the culture vessel. It exhibits elaborate surface projections that characterize many heavily confluent cells.  $\times 12,500$ . (B) Another confluent cell which has been exposed to 1 mg/ml HRP for 60 min before washing and cytochemical localization of enzyme. In addition to the granules and vesicles that are found in rapidly growing cells, confluent ones occasionally exhibit much more complex structures lined by a rim of reaction product (arrows). The interior of such structures is pierced by many cytoplasmic infoldings. Conceivably they arise by fusion of deep surface invaginations which in turn may contain cytoplasmic protrusions (\*).  $\times 15,000$ .



particulate immune complexes of HRP, with either mouse or rabbit antiHRP, enhanced the rate of uptake of enzyme several thousandfold. This was attributed to the presence of a membrane receptor on macrophages that recognized the Fc region of the immunoglobulin in immune aggregates. L cells can phagocytose nonimmune particulates (44), but they were unable to bind and interiorize aggregates of HRP-antiHRP, using both cytochemical and quantitative assays.

### Metabolic Parameters of Pinocytosis

The logarithm of the rate of pinocytosis in L cells increased linearly with the temperature of the incubation medium (Fig. 9). The  $Q_{10}$ , i.e., the increase per  $10^{\circ}\text{C}$  rise in temperature, is 2.7-fold. The amounts of HRP bound to L-cell monolayers at low temperatures ( $10^{\circ}\text{C}$  or less) was very small, and intracellular HRP was more difficult to visualize cytochemically. However, we assume that the same process, i.e., pinocytosis, was in fact being quantitated at all temperatures studied, since the data fit a single exponential without any break in the plot over the entire  $2\text{--}38^{\circ}\text{C}$  range studied. When the logarithm of the pinocytic rate is plotted against the reciprocal of the incubation tempera-

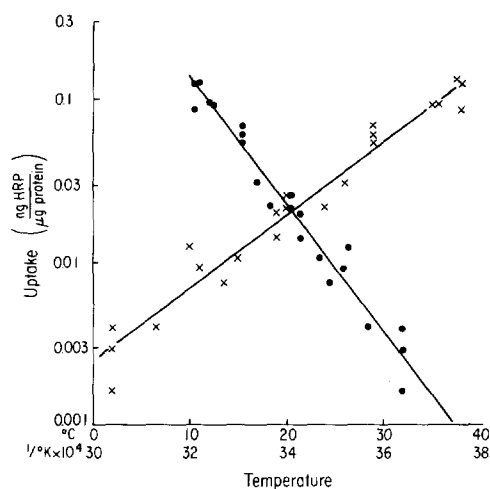


FIGURE 9 Effect of temperature on pinocytosis. The logarithm of the rate of pinocytosis increases linearly with temperature in degrees Centigrade ( $\times-\times$ ). The  $Q_{10}$  defined by the slope is 2.7. When the rate is plotted against the reciprocal of the temperature in degrees Kelvin ( $\text{O}-\text{O}$ ), an Arrhenius plot is obtained with an activation energy, defined by the slope, of 17.8 kcal/mol. The correlation with which these data fit a straight line is 0.97.

TABLE II  
Reversal of the Increased Pinocytic Activity of Confluent Cells

Time after replating confluent cells	Uptake of 1 mg/ml HRP load	Cell protein
	ng/ $\mu\text{g}$ protein/h	$\mu\text{g}/60\text{-mm}$ dish
2	0.218	165
4	0.198	172
6	0.229	183
10	0.185	217
18	0.136	304
24	0.118	400
32	0.105	575

Confluent cultures were trypsinized and replated as sparse cultures ( $6 \times 10^5$  cells/60-mm dish). At varying time points thereafter, the monolayers were assayed for pinocytic activity and cell protein. The replated cells quickly resume the level of rapid growth characteristic of sparse cultures, as evidenced by the increase in cell protein per culture. The increased pinocytic activity of confluent cells persists for hours and does not fully return to the level characteristic of growing cells for 24 h or more.

ture in degrees Kelvin (Fig. 9), an Arrhenius plot is obtained in which the slope defines the energy of activation of pinocytic uptake. This value is 17.9 kcal/mol.

From previous studies on phagocytosis (reviewed in reference 32) it can be presumed that a critical temperature-dependent step in pinocytosis involves the generation of energy as ATP. L cells were therefore treated with four agents known to diminish ATP supplied by glycolysis (2-deoxyglucose, sodium fluoride) or respiration (sodium cyanide and sodium azide). The drugs were applied for 1 h in fresh culture medium, and then HRP was added to a final concentration of 1 mg/ml for 1 additional h in the presence of inhibitor. Three parameters were then evaluated: uptake as determined by enzymatic assay; distribution of cell-bound HRP assessed cytochemically; and cellular ATP levels measured in a luciferin-luciferase assay. None of the drugs diminished cell numbers during the 2-h exposure, and the cells resumed growth normally after their removal from the culture medium.

All four agents were able to inhibit HRP uptake. We first established the doses that would produce

maximum inhibition (Table III). This was  $10^{-2}$  M for NaF,  $5 \times 10^{-2}$  M for 2-DG (a ninefold excess of the glucose concentration in the culture medium), and  $10^{-3}$  M for cyanide and azide. An additional two- to fivefold increase in the concentration of the drugs above these levels did not further diminish the uptake of HRP. This suggests that the inhibition observed was due to a block in a selected process, e.g., energy metabolism, rather than some generalized toxic effect.

Additional studies showed that the inhibitors were in fact decreasing the rate of pinocytosis. Determination of uptake after varying times of HRP exposure showed that inhibited cells pinocytosed at a reduced level throughout the 1-h assay period (Fig. 10). Cytochemically, cell-bound HRP was distributed in granules accumulating in the Golgi zone, i.e., as in normal pinocytosing cells (Fig. 11 A, B). By both light and electron microscope examination, it was difficult to distinguish between normal and inhibited cells in terms of the number and localization of peroxidase-containing granules.

We tentatively concluded that energy derived from either glycolytic or respiratory pathways drives pinocytosis in L cells. When combinations of drugs were used to block both sources of metabolic energy, further inhibition of HRP uptake was observed (Table IV). Pinocytic activity

TABLE III  
Dose Response of Drug-Induced Inhibition of HRP Uptake

Concentration of inhibitor (M)	Inhibition of pinocytosis by			
	NaF	2-DG	NaN <sub>3</sub>	NaCN
	%	%	%	%
$10^{-1}$	—	42	—	—
$5 \times 10^{-2}$	—	40	—	—
$2 \times 10^{-2}$	63	10	—	—
$10^{-2}$	65	0	—	—
$5 \times 10^{-3}$	53	—	44	38
$10^{-3}$	35	—	43	38
$5 \times 10^{-4}$	10	—	30	20
$10^{-4}$	0	—	10	8
$5 \times 10^{-5}$	—	—	0	0

Growing L cells were exposed for 1 h to varying concentrations of metabolic inhibitors in 5% FCS-MEM. HRP was then added for an additional hour at a concentration of 1 mg/ml. Uptake was assessed by enzymatic assay and compared with control cells.

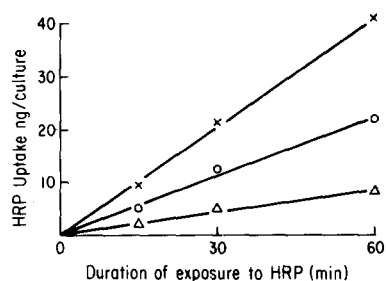
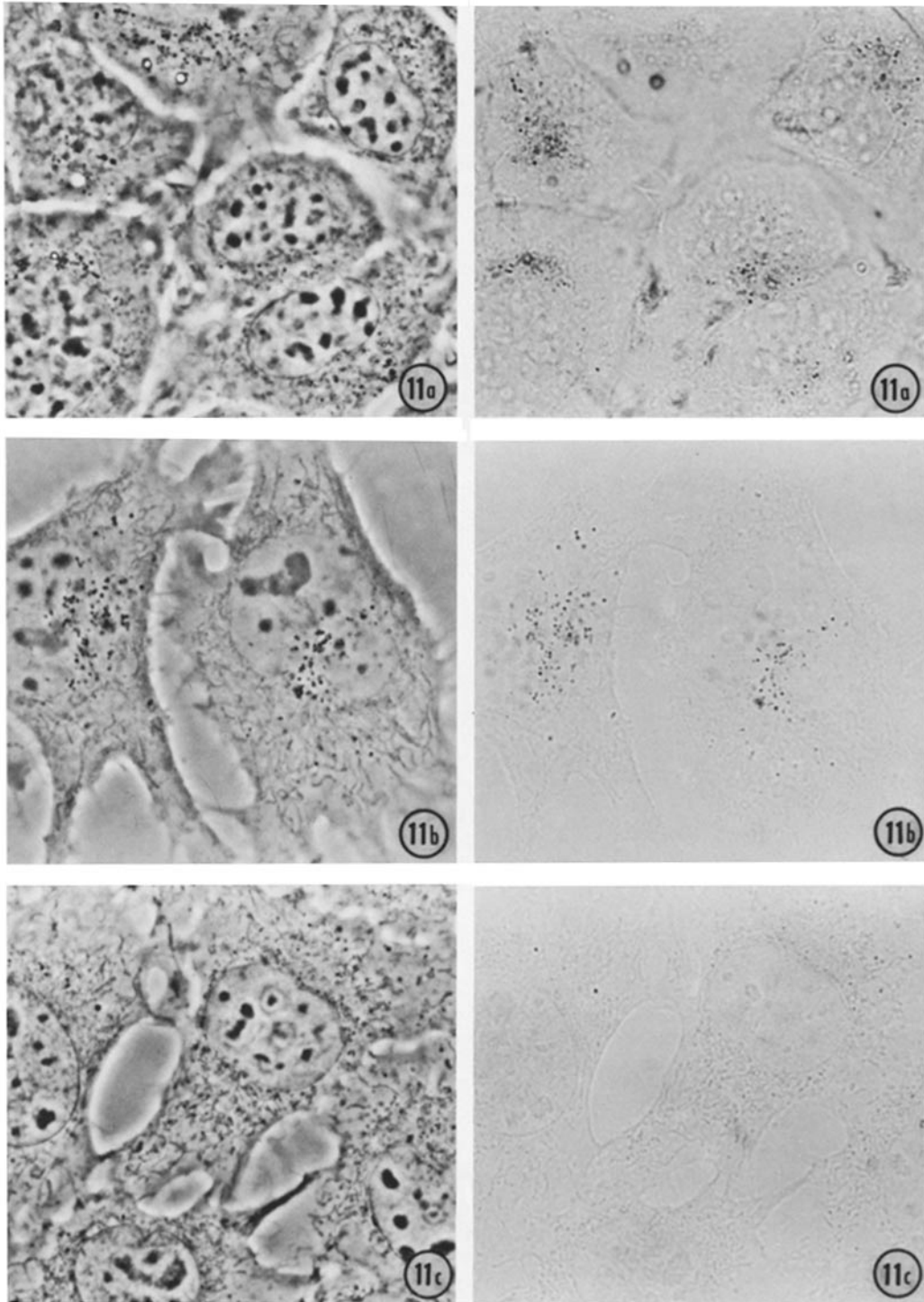


FIGURE 10 HRP uptake in the presence of metabolic inhibitors. The rate at which control cells pinocytose HRP ( $\times-\times$ ) is diminished in the presence of  $10^{-2}$  M NaF ( $\circ-\circ$ ), or the combination of  $5 \times 10^{-2}$  M 2-DG and  $10^{-3}$  M NaN<sub>3</sub> ( $\Delta-\Delta$ ).

was reduced about 80% but never totally abolished. In contrast, the simultaneous administration of two respiratory or two glycolytic inhibitors did not enhance the inhibition of HRP uptake over either drug alone. Cell monolayers treated with combinations of respiratory and glycolytic inhibitors contained little or no reaction product, as assayed by light microscope cytochemistry (Fig. 11 C). However, in thin sections, HRP-positive structures were found, suggesting that the residual uptake quantitated in the enzymatic assay did in fact represent enzyme interiorized by endocytosis.

Direct measurements of cellular ATP levels substantiated the possibility that energy metabolism was in many instances being blocked in the drug-treated cultures (Table V). 2-DG, NaF, and NaCN administered singly produced a 30–60% fall in cellular ATP, but azide had little or no effect. Any combination of a glycolytic and a respiratory inhibitor (including azide), however, resulted in a further drop, i.e., to 10–20% of the control cellular ATP values. The decreases in ATP levels were maximal within 15 min after addition of the inhibitors.

The effects of the four inhibitors were examined in confluent as well as growing cultures. Untreated confluent cultures pinocytosed more actively than their growing counterparts (see above) although their ATP levels were lower (Table V). Both azide and cyanide decreased HRP uptake in confluent cells, but again, azide did not produce a significant drop in cellular ATP. 2-deoxyglucose did not block HRP uptake in confluent cells even though it diminished ATP levels to a greater extent than in growing cultures. Fluoride, in contrast, did decrease both parameters in confluent cultures. This



**FIGURE 11** Light microscope localization of HRP in control and drug-treated L cells. Phase-contrast (left) and bright-field (right) micrographs were taken of L cells exposed to 1 mg/ml HRP for 1 h and processed cytochemically. (A) Control cells exhibit many perinuclear granules with enzyme.  $\times 2,800$ . (B) Cells treated with  $5 \times 10^{-2}$  M 2-DG, before and during exposure to HRP, exhibit a similar number and localization of reactive granules as seen in controls. Biochemically, 2-DG measurably (40%) diminished HRP uptake.  $\times 2,900$ . (C) Cells treated with a combination of 2-DG and NaCN contain little reaction product at the light microscope level, though in thin sections, small numbers of membrane-bound structures containing enzyme can be visualized. Biochemically, uptake of HRP was decreased by 80% of the control.  $\times 2,750$ .

suggested that fluoride may be able to inhibit pinocytosis by some other means than blocking glycolysis. We therefore tried to reverse its effect by adding 5 mM sodium pyruvate to the culture medium. It has been suggested that if glycolysis is the only process blocked by a drug, then some compensation of ATP levels in inhibited cells might be achieved with the increased availability of citric acid cycle substrates (49, 63). Pyruvate did not reverse the inhibition of HRP uptake by fluoride, but in growing cells it did reverse the effects of 2 DG (70–100% in six experiments).

## DISCUSSION

### *HRP as a Marker for Pinocytosis*

Horseshoe peroxidase appears to be an excellent marker with which to study the ongoing pinocytosis of fluid and solutes in tissue culture cells, such as fibroblasts (this study) and macrophages (53). A critical requirement for any marker solute is that it be taken up in the fluid phase, without adsorption to the cell surface. The level of HRP uptake is directly proportional to the concentration of enzyme in the medium, over a wide range of solute concentrations. At any given concentration, uptake proceeds linearly with time for at least 3 h. Lowering the environmental temperature dramatically lowers the binding of HRP to cells. Cytochemically, HRP reaction product was never visualized attached to the cell surface, but in cells exposed to enzyme for just 5–10 min, it was easily localized in small intracellu-

lar vesicles. These features of HRP uptake prove that it is being interiorized in the fluid phase and are clearly different from the characteristics of uptake of materials which bind to the cell surface before engulfment. Adsorptive pinocytosis has been best studied in amoebae (11, 12, 29, 50), and generally leads to a more extensive uptake rate than that recorded for solutes taken up in the fluid phase. Adsorptive uptake is easily saturated with increasing concentrations of solute in the medium,

TABLE IV  
*Effects of Metabolic Inhibitors on L Cell Pinocytosis*

Inhibitor(s)	Inhibition of HRP uptake
	%
$10^{-2}$ M NaF	47
$5 \times 10^{-2}$ M 2-DG	35
$10^{-3}$ M NaCN	28
$10^{-3}$ M $\text{NaN}_3$	31
NaF + NaCN	80
NaF + $\text{NaN}_3$	77
2-DG + NaCN	84
2-DG + $\text{NaN}_3$	83
NaF + 2-DG	49
NaCN + $\text{NaN}_3$	30

Preconfluent L cells were exposed for 1 h to one or two metabolic inhibitors in 5% FCS-MEM. 1 mg/ml HRP was then added for an additional hour. The values given are for a single representative experiment obtained on the same set of replicate cultures.

TABLE V  
*Effects of Metabolic Inhibitors on L Cell Monolayers*

Inhibitor	Preconfluent cells		Confluent cells	
	Decrease in HRP uptake	Decrease in cellular ATP	Decrease in HRP uptake	Decrease in cellular ATP
	%	%	%	%
$10^{-2}$ M NaF	43 ± 17	35 ± 9	62 ± 10	45 ± 5
$5 \times 10^{-2}$ 2DG	41 ± 13	34 ± 6	9 ± 5	60 ± 7
$10^{-3}$ M NaCN	30 ± 10	27 ± 7	31 ± 6	38 ± 10
$10^{-3}$ M $\text{NaN}_3$	30 ± 12	3 ± 3	43 ± 14	5 ± 9
2DG + NaCN	75 ± 4	88 ± 3	85 ± 4	82 ± 4
2DG + $\text{NaN}_3$	78 ± 11	92 ± 4	86 ± 6	83 ± 3

The data are means and standard deviations of 4–10 determinations. Preconfluent monolayers had 300–700  $\mu\text{g}$  protein (per 60-mm dish) and confluent cells had 800–1,400  $\mu\text{g}$ . ATP levels per  $10^6$  cells were 3.0–4.5 nmol for preconfluent cells, and 1.7–2.6 nmol for confluent ones.

and does not proceed linearly for long periods of time. The initial binding of solute to the cell occurs at low temperatures and can also be demonstrated directly by morphological techniques (6, 7, 37).

The use of HRP is facilitated by several features which make it, we think, the most advantageous marker currently available. Excellent preparations can be obtained commercially, and results are similar to those obtained with highly purified materials (53). The enzyme is readily soluble in tissue culture media, is nontoxic, and behaves as a uniform preparation when the same solution is tested repeatedly on successive cell monolayers. Like other proteins, it binds to the surface on which cell monolayers are maintained, but this dish-bound compartment can be eluted by an appropriate washing procedure. Quantitative measurements of uptake can be made in cell lysates with a relatively simple and reliable (less than 5% standard deviation of triplicate measurements) enzymatic assay which detects as little as 1 ng/ml of HRP in a cell lysate. Last, the Graham and Karnovsky cytochemical procedure (24) permits reliable light and electron microscope localization of HRP to the vesicles and granules of the vacuolar apparatus. In fact, current quantitative studies suggest that all incoming pinocytotic vesicles are detectable in the electron microscope with this technique.

Other test substances have been used for quantitative work on pinocytosis, although usually detailed studies on the effects of solute concentration, exposure time, temperature, dish binding, and cell number are not reported. Proteins, either radiolabeled (21, 46, 47) or enzymatically active (2, 15, 57) are often employed. Uptake data with serum albumins in both macrophages (21) and fibroblasts (46, 47) are of a similar order of magnitude to those reported for HRP. However, this marker would not seem useful for short term quantitative work. The variability in replicate measurements may be considerable, and uptake may be obscured by the adsorption of large amounts to the culture vessel. Radiolabeled small colloidal particles, especially colloidal gold, have also been used as pinocytotic markers (15, 23, 60). These preparations may vary considerably in the degree to which they are interiorized by adsorptive vs. fluid mechanisms. In addition, colloidal gold may adhere to the culture vessel, especially in the presence of low serum concentrations (Z. A. Cohn, unpublished observations). Finally, [ $^3\text{H}$ ]sucrose may be a suitable test material. This disaccharide does not ap-

pear to permeate cell membranes and clearly enters the vacuolar system (16, 58). Quantitative work has been hampered by rather high background levels of uncertain etiology, i.e., as much as 50% of the total binding in 1 h can be accounted for as background "uptake" (4, 58). Also, it is still uncertain at what rate sucrose may escape the vacuolar confines and if sucrose can be degraded by cells. For example, sucrose-laden vacuoles in macrophages disappear spontaneously in apparently healthy cells cultivated overnight (Z. A. Cohn, unpublished observations).

### *The Influence of Cell Density on Pinocytosis*

L-cell monolayers maintained at high density after reaching confluence pinocytosed more actively (up to fourfold) than rapidly growing pre-confluent cells. The enhanced rate of HRP uptake could not be attributed to some factor in the culture medium. In contrast, confluent cultures of 3T3 cells and calf embryo fibroblasts did not increase their pinocytotic activity. L cells are distinctive in that they continue to grow on reaching confluence and occupy less and less of the culture vessel's surface area. The cell surface becomes extremely irregular and exhibits many projections (ruffles, blebs) as well as invaginations. The latter are unusual in mammalian cells (with the possible exception of the vermiform invaginations of Kupffer cells [reviewed in reference 62]), but are frequently found in amoeba where they participate in pinocytosis (6, 29, 37). Lewis observed the association of pinocytotic vesicle formation with areas of cell surface activity, but it is not clear if the enhanced pinocytotic activity of confluent L cells is the cause or the result of the altered surface morphology. Several other etiologies are of course possible. L cells may pinocytose more actively in the  $G_1$  phase of their cell cycle, which is the predominant stage at which confluent cells are found. The influence of cell-to-cell contacts, nutritional requirements, and altered energy metabolism on pinocytotic activity of confluent cells is also unknown.

### *Comparison of Mouse Fibroblasts and Macrophages*

Three different continuous mouse fibroblast lines, as well as recently explanted calf embryo



fibroblasts, all interiorize HRP at similar rates—10<sup>-5</sup>% of the administered load per microgram cell protein per hour. The significance of this constancy among the various fibroblasts is unclear, but on a unit of cell protein basis, their pinocytotic activity is 10 times less than that of macrophages from unstimulated mouse peritoneal cavities (53). The fibroblasts we studied contain 10 times as much protein per cell, so that on a per cell basis, the pinocytotic activity of fibroblasts and macrophages in vitro is quite similar.

In both cell types, exogenously added HRP is restricted in distribution to the vacuolar apparatus, where its enzymatic activity disappears completely and in an exponential fashion at identical rates, the  $t_{1/2}$  being about 7 h. We interpret this similarity to indicate that the lysosomes in both types of mouse cell are similar, i.e., in the nature and content of enzymes capable of inactivating HRP and in the rate at which they are delivered to incoming HRP-containing vesicles. At this time, however, the presumptive hydrolase(s)-destroying HRP activity has not been identified. We also found that HRP pinocytosed by L cells is not detectably exocytosed into the culture medium. A similar lack of exocytosis of HRP (53) and other markers (15) in macrophages has been documented.

The most impressive difference in the macrophage and fibroblast as endocytic cells in vitro appears not to involve pinocytosis but rather phagocytosis of particulates, especially those coated with antibody. Macrophages possess an  $F_c$  receptor activity which allows them actively to bind and interiorize particles coated with immunoglobulin G, e.g., particulate HRP-antiHRP aggregates at equivalence (54), whereas fibroblasts clearly lack this capacity as indicated by using either the HRP-antiHRP marker (this study), or antibody-coated red cells (43).

#### *Effect of Temperature*

In this study, the principle application of HRP as a marker solute was to study the metabolic parameters of pinocytosis in L cells. Pinocytosis is an endothermic phenomenon in which the  $Q_{10}$  over a wide range of temperatures (2–38°C) is 2.7, corresponding to an activation energy of 17.6 kcal/mol. We failed to find a temperature at which the activation energy for pinocytosis was sharply altered. Our data are insufficient to rule out the existence of a relatively small change in activation energy (31). The lack of a distinct “transition temperature” for pinocytosis is apparently in con-

trast to a number of other activities of the cell surface, e.g., respiration, growth, and transport in bacteria (41, 61), agglutinability of transformed tissue culture cells by lectins (38), ATPase activity in lamb kidney (25), and exocytosis of histamine from mast cells (33). The existence of these biological transition temperatures is attributed to a phase change in the membrane lipids, analogous to the transition temperatures detected by a variety of physicochemical criteria in lipids and membranes (reviewed in references 10 and 51). It is not known if a true transition temperature can be detected in membranes of L cells over the range of 2–38°C that we studied, but if one does exist, it would not appear to influence the cells's ability to form pinocytotic vesicles. This would not be surprising since the presumably bulk movements of membrane during pinocytosis may be very different from the movement and/or activity of functional moieties within the membrane.

The precise meaning of an activation energy of 17.6 kcal/mol for pinocytosis is not clear. Activation energies for a variety of biological processes in whole tissues and organisms have been catalogued (reviewed in reference 31) and fall within a range of 4–34 kcal/mol. Similar values have been obtained more recently for more restricted cellular activities (25, 26, 33, 41, 59, 61). A number of physical and chemical changes are probably involved in pinocytosis, and the activation energy must reflect the requirements of the rate-limiting process. But the latter is entirely unknown and could include such aspects as the production of energy needed for pinocytosis, alterations in the viscosity of membrane lipids (18), or the function of a contractile mechanism (60).

#### *Effects of Metabolic Inhibitors*

The metabolic requirements for endocytosis have frequently been studied through the use of inhibitors. We concentrated on agents presumed to block energy production, since this approach has been used profitably to show that cellular ATP was required for phagocytosis. This ATP could be generated either by *glycolysis*, e.g., in most normal neutrophilic leukocytes (48), or by *respiration*, e.g., in alveolar macrophages (40), and in neutrophils unable to glycolyze because of an enzymatic defect (3) or the presence of a glycolytic inhibitor (49). A consistent picture for pinocytosis has not yet emerged although morphological methods primarily have been used, e.g., vesicle counts in the light microscope (13), or detection of appropriate markers in the electron microscope (8). It has even

been suggested that pinocytic vesicles can be interiorized in the absence of metabolic energy (8).

The L cells we studied generated ATP by both glycolysis and respiration. In contrast, the 3T3 fibroblasts studied by Vlodavsky et al. were almost entirely dependent on respiration for their content of ATP (56). Combinations of drugs that inhibited both pathways were required to depress L-cell ATP levels to 10–20% of control values. The failure to inhibit either parameter completely could be due to residual glycolytic or respiratory activity, and/or to some other supply of ATP, e.g., via creatine phosphate or substrate phosphorylation. In any case, the severe depletion of cellular ATP was associated with a similar 80–90% inhibition of HRP uptake.

Studies with single metabolic inhibitors produced more complicated findings. The average data from several experiments indicated that most of the agents produced a 30–50% fall in both cellular ATP levels and HRP uptake. However, several exceptions were noted. First, in any one experiment, the decrease in ATP vs. HRP uptake could be quite different. Second, azide by itself did not lower ATP levels but did diminish pinocytosis, so that azide can presumably influence pinocytosis in some other way. Likewise, fluoride blocked pinocytosis, but this effect could not be reversed by addition of substrate (pyruvate) to enhance production of ATP via respiratory means (49). Finally, 2-deoxyglucose substantially decreased ATP levels (60%) in confluent L cells but did not block pinocytosis. Conceivably, the threshold level of ATP required for pinocytosis in confluent cells is really quite low, or there is possibly a restricted compartment of ATP that is utilized. At a first glance, then, the observations with single inhibitors would not support a one-to-one relationship between ATP levels and HRP uptake. But clearly the system is a complex one in which we are lacking information on the stores of substrate in L cells, the penetrability of the various agents, other possible effects of the drugs, etc.

### *Functions of Pinocytosis*

Although the HRP system has provided a good deal of quantitative information on the pinocytic process *in vitro*, the functional significance of pinocytosis *in vitro*, and *in vivo* in many cell types, is not clear. Lewis (34) felt that pinocytosis served a nutritional role in mammalian cells. From our data on growing fibroblasts, it is obvious that pinocytosis of proteins, followed by digestion in lysosomes to appropriate building blocks, is not serving a bulk nutritive function. The rate of increase in cell protein (doubling time of 22 h) far exceeds the influx of exogenous protein from the

environment. Medium with 5% fetal calf serum contains about 3 mg/ml of protein; therefore, 1  $\mu$ g of cell protein, pinocytosing at a rate of  $10^{-5}$ % of the administered concentrations per hour per microgram cell proteins would interiorize only  $10^{-2}$   $\mu$ g during the time it doubles in protein content. Eagle and Piez (20) obtained direct evidence that exogenous macromolecules did not provide building blocks for growth of cells in culture, when they showed that radiolabel in exogenous proteins was not incorporated to any significant extent (less than 3–6%) in the newly synthesized proteins of the cell. However, in both Eagle's and Piez's and our study, cells were maintained in media replete with small molecule nutrients which they readily transport directly across the cell surface. It would be interesting to find out if media deficient in certain nutrients could be restored by macromolecules, and if such a deficiency resulted in a stimulation of pinocytic activity. It is also possible that certain low molecular weight nutrients require pinocytosis for entry into the cell. A small molecule might be bound in the environment to a large molecular carrier; the latter would enter the cell by pinocytosis, be digested, and release the nutrient into the cytoplasm. However, physiological examples of such an event are not known. Many amoebae fail to transport sugars and amino acids across their cell surfaces but do obtain these small molecular weight nutrients via pinocytosis (5, 11, 12, 29). Fusion with lysosomes probably brings about an alteration of the vacuolar membrane, which, though derived from the apparently impermeable plasma membrane, now permits transport of the nutrient into the cytoplasm.

Rather than focusing on the content of pinocytic vesicles, it may be fruitful to consider the cell membrane surrounding these vesicles. It has been suggested that in many secretory cells, cell membrane added to the cell surface during the exocytosis of membrane-bound secretory vesicles is returned to the intracellular space by pinocytosis (e.g., see references 1, 9, 19, 27, and 39). Quantitative proof of this hypothesis has not yet been obtained, though its occurrence at least in qualitative terms is being frequently documented. We are currently quantitating the portion of the cell surface area which may be interiorized as pinocytic vesicle membrane per hour. We have been impressed that L cells may interiorize the equivalent of 25% or more of their surface area each hour. It is possible that the function of the pinocytic process involves this large influx of membrane, e.g., allowing cells to restore and remodel their plasma membrane, as well as to reutilize and recirculate components used in its formation. Such studies will be presented in a subsequent communication.

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