Phorbol Esters and Horseradish Peroxidase Stimulate Pinocytosis and Redirect the Flow of Pinocytosed Fluid in Macrophages

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ABSTRACT Lucifer Yellow CH (LY) is an excellent probe for fluid-phase pinocytosis. It accumulates within the macrophage vacuolar system, is not degraded, and is not toxic at concentrations of 6.0 mg/ml. Its uptake is inhibited at 0°C.

Thioglycollate-elicited mouse peritoneal macrophages were found to exhibit curvilinear uptake kinetics of LY. Upon addition of LY to the medium, there was a brief period of very rapid cellular accumulation of the dye (1,400 ng of LY/mg protein per h at 1 mg/ml LY). This rate of accumulation most closely approximates the rate of fluid influx by pinocytosis. Within 60 min, the rate of LY accumulation slowed to a steady-state rate of 250 ng/mg protein per h which then continued for up to 18 h. Pulse-chase experiments revealed that the reduced rate of accumulation under steady-state conditions was due to efflux of LY. Only 20% of LY taken into the cells was retained; the remainder was released back into the medium. Efflux has two components, rapid and slow; each can be characterized kinetically as a first-order reaction. The kinetics are similar to those described by Besterman et al. (Besterman, J. M., J. A. Airhart, R. C. Woodworth, and R. B. Low, 1981, *J. Cell Biol.* 91:716–727) who interpret fluid-phase pinocytosis as involving at least two compartments, one small, rapidly turning over compartment and another apparently larger one which fills and empties slowly.

To search for processes that control intracellular fluid traffic, we studied pinocytosis after treatment of macrophages with horseradish peroxidase (HRP) or with the tumor promoter phorbol myristate acetate (PMA). HRP, often used as a marker for fluid-phase pinocytosis, was observed to stimulate the rate of LY accumulation in macrophages. PMA caused an immediate four- to sevenfold increase in the rate of LY accumulation. Both HRP and PMA increased LY accumulation by stimulating influx and reducing the percentage of internalized fluid that is rapidly recycled. A greater proportion of endocytosed fluid passes into the slowly emptying compartment (presumed lysosomes). These experiments demonstrate that because of the considerable efflux by cells, measurement of marker accumulation inaccurately estimates the rate of fluid pinocytosis. Moreover, pinocytic flow of water and solutes through cytoplasm is subject to regulation at points beyond the formation of pinosomes.

It is established that fluid-phase pinocytosis occurs by the interiorization of plasma membrane as vesicles. The fluid trapped in these vesicles, or pinosomes, is delivered to lysosomes. Several types of mammalian cells are capable of internalizing a large volume of medium by this method. Steinman et al. (14) have calculated that murine resident peritoneal macrophages internalize the equivalent of their own cell surface area in pinosomal membrane every 33 min and 25% of their own volume in fluid per hour. Since this considerable influx is a continuous process, and since cells such as macrophages maintain fairly constant dimensions, there must be some return flow. Internalized membrane and protein receptors therein have been shown to return to the cell surface, presumably in vesicle form (16). Pinocytosed fluid presents a

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 MARCH 1985 851–859
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different problem. The continuous gulping of medium brings into the cytoplasm not only nutritive solutes but also Na^+, which most metazoan cells work hard to keep outside. Excretion of these ions could occur either in vesicle form by returning pinosomes to the cell surface or by ion-pumping across lysosomal or plasma membranes. The contributions of these alternative pathways to the regulation of water balance and net flow of solutes in cells have not been determined.

Fluid-phase pinocytosis is usually studied by measuring the cellular accumulation of a soluble and impermeant probe. Steinman and Cohn (15) used horseradish peroxidase (HRP)[1] as a fluid-phase marker in fibroblasts and peritoneal macrophages. They found that accumulation is linear for several hours and that there is no appreciable exocytosis. It has since been shown that cells containing surface mannose-receptors pinocytose HRP by adsorptive mechanisms (6), and a large percentage of HRP uptake by thioglycollate-elicited mouse peritoneal macrophages (thio-macrophages) is via these receptors (18). Therefore, uptake of HRP reflects both adsorptive and fluid-phase pinocytosis in these cells.

Besterman et al. (4) used [^{14}C]sucrose as a probe for pinocytosis. They found accumulation to be nonlinear, with a considerable efflux of [^{14}C]sucrose and fluid from cells. Using kinetic evidence they proposed that accumulation is the sum of several processes, which include the filling of two different compartments and exocytosis from both.

In this paper we examine the regulation of pinocytic flow through cytoplasm and try to resolve apparent contradictions between the results of Steinman and Cohn (15) and Besterman et al. (4). Are the kinetics of probe accumulation linear or curvilinear?

Miller et al. (8) used Lucifer Yellow (LY) to label lysosomes and suggested that it reaches those organelles by pinocytosis. Here we describe LY as a probe for fluid-phase pinocytosis and use it to analyze the constitutive pinocytic flow into and through cells. We show that accumulation is curvilinear during the first hour of uptake, then linear thereafter. Furthermore, we find that accumulation misrepresents pinocytic rate, as a considerable efflux of fluid and solutes accompanies pinocytosis. Finally, we examine the effects of HRP or the tumor promoter phorbol myristate acetate (PMA) on LY influx and efflux.

MATERIALS AND METHODS

Cells: Female, white mice (ICR, Trudeau Institute, Saranac Lake, NY; or NCS, The Rockefeller University) were injected with thioglycollate broth 4 d before harvest. Mice were killed by CO_2 asphyxiation. Their peritoneal cavities were rinsed with 5 ml cold, divalent cation-free, phosphate-buffered saline (PD); then the cells suspended in this PD were washed twice by centrifugation. 6–8 $\times 10^5$ peritoneal cells in 1 ml of Eagle's minimum essential medium with 10% heat-inactivated fetal bovine serum (M10F) were plated into each well of 24-well Costar dishes (Costar, Data Packaging, Cambridge, MA). After 2 h at 37°C, dishes were washed with cold PD to remove nonadherent cells. 1 ml of M10F was added to each well, and these cultures were maintained at 37°C in a 5% CO_2 atmosphere. Experiments were carried out 18–24 h after harvest of peritoneal cells.

Measurement of Pinocytosis: Lucifer Yellow CH (reference 17) was dissolved in M10F at 0.1–1.0 mg/ml (usually 0.5 mg/ml) and then filtered just before the experiment. Cells were incubated with 0.45 ml LY-containing medium per Costar well for various periods of time. Dishes were drained and then immersed sequentially, first in two 1-liter volumes of PD with 1 mg/ml BSA, then in two 1-liter volumes of PD, all at 4°C. The wells were drained again, and 0.50 ml Triton X-100 (0.05%) was added to each well to lyse cells. Adherent cellular debris was scraped from wells with a rubber policeman.

To measure fluorescence, 0.35 ml of lysate was brought to 1.60 ml in 0.05% Triton X-100 containing 0.1 mg/ml BSA (Triton/BSA). Fluorescence was measured on a Perkin-Elmer MPF-44A fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) with excitation at 430 nm (bandwidth = 10 nm) and emmission at 540 nm (bandwidth = 18 nm). Standard curves of LY were prepared in Triton/BSA and were found to be linear from 0.1 to 100 ng/ml and could resolve 0.05 ng LY. (Since LY binds to glass, it was necessary to prepare standard curves using plasticware and medium containing 0.1 mg/ml BSA.)

Protein was determined using the method of Lowry et al. (7). As Triton caused a flocculent precipitate to form in the samples used for protein determination, we centrifuged samples (300 *g*) before reading at 750 nm. Protein standards were prepared in 0.025% Triton from BSA stock (1 mg/ml) and similarly spun before reading.

Uptake was calculated for each well as nanograms of LY per milligram of protein. Each time point was done in duplicate or triplicate and expressed as the mean ± SD. The apparent fluorescence of lysate from cells unexposed to LY was subtracted from other fluorescence values to determine the cell-associated fluorescence of LY.

Pulse-Chase Experiments: To measure rates of efflux, cells in Costar wells were incubated in LY for various times and washed in PD/BSA and PD as described above. 0.25 ml of cold M10F (plus 10 mM HEPES, pH 7.2) was added to each well, and the cells were then incubated at 37°C for a "chase" period. Each time point of chase required a separate dish of cells. At the end of the chase period M10F was aspirated from the wells, the dish was rinsed once more in ice-cold PD, and the cells were lysed in 0.50 ml Triton X-100 (0.05%).

Microscopy: Cells were plated at 37°C onto 12-mm-diam circular coverslips (7.5×10^4 cells per coverslip), rinsed, and cultured similarly to those in Costar wells. Macrophages were incubated for various periods with 1 mg/ml LY in M10F, rinsed with cold PD, then observed either living or after fixation with 3.7% formaldehyde in PD. Fixed cells were rinsed with PD, mounted in glycerol, and immediately examined with a 100× oil-immersion lens in a Zeiss Photomicroscope III equipped with fluorescence optics (fluorescein filter set). Cells were photographed using Tri-X film (ASA set for 3200).

Other Methods: Chromatography was carried out using Sephadex G-25 (Pharmacia Inc., Piscataway, NJ; fine grade) in a 20-cm column (bed volume = 7 ml). Cell viability was measured with 0.1% trypan blue in PD. HRP was measured using H_2O_2 and *o*-dianisidine according to Steinman and Cohn (15) and Sung et al. (18). PMA was dissolved in dimethylsulfoxide at a stock concentration of 0.3 mg/ml and was added to cells at a final concentration of 10 ng/ml in M10F. Phorbol dibutyrate was dissolved in dimethylsulfoxide at 3 mg/ml and was added to cells at 500 ng/ml. HRP, LY, and PMA were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Thioglycollate-elicited mouse peritoneal macrophages (thio-macrophages) in medium that contained LY continually accumulated the fluorescent probe. The rate of accumulation was initially rapid but after 60 min reached a slower, steady-state rate that remained constant thereafter (250 ± 43 ng/mg protein per h at 1.0 mg/ml; Fig. 1*A*). Longer incubations in LY revealed that the steady-state rate could continue for 18 h, after which accumulation was reduced (Fig. 1*B*). Very little uptake occured at 0°C. Metabolic inhibitors such as KCN and 2-deoxyglucose slowed but did not stop accumulation (Fig. 1*A*).

LY was not toxic to the macrophages. More than 95% of cells incubated for 6 h in medium containing 6 mg/ml LY or for 72 h in medium with 0.3 mg/ml LY excluded trypan blue, and there was no change in their morphology as observed by phase-contrast microscopy.

Cells pulsed 5 min with LY, washed, fixed with formaldehyde, and then observed by fluorescence microscopy exhibited punctate fluorescence near the cell periphery (Fig. 2*A*). Longer incubations (60–120 min) yielded additional fluorescence in

[1] *Abbreviations used in this paper:* LY, Lucifer Yellow CH; HRP, horseradish peroxidase; PMA, phorbol myristate acetate; PD, divalent cation-free phosphate-buffered saline, M10F, minimum essential medium with 10% heat-inactivated fetal bovine serum; thio-macrophages, thioglycollate-elicited mouse peritoneal macrophages.

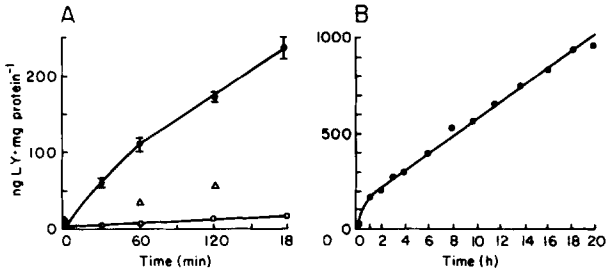

FIGURE 1 (*A*) Pinocytosis of LY by thio-macrophages. Cells were incubated in 0.3 mg/ml LY in M10F for various times. ●, 37°C; △, 37°C with 50 mM 2-deoxyglucose and 1 mM KCN; ○, 0°C. (*B*) Long time-course of pinocytosis. Cells were incubated in 0.3 mg/ml LY for the indicated times, washed, and lysed.

larger vacuoles, presumably the inclusions characteristic of thio-macrophages (Figs. 2, *B* and *C*). When live cells were examined after long incubations, the fluorescent compartments often appeared string-like or tubular. Addition of fixative caused fragmentation of the tubules into punctate vesicles. Cells pulsed for one hour and chased for two before fixation exhibited a noticeable reduction in the finely particulate peripheral fluorescence with little change in the fluorescence of the larger LY-containing vacuoles. The LY was always in discrete compartments, and there was no indication of LY diffusion into the cytoplasm.

Uptake Is Not Adsorptive

Accumulation was directly proportional to the probe concentration; cells in 1.0 mg/ml LY accumulated 10 times as much as cells in 0.1 mg/ml LY (Fig. 3). This observation, coupled with the observed cessation of uptake at 0°C, suggested that there was no adsorptive component to uptake. When LY (1 mg/ml) was added to cells and immediately removed (0-time incubation), very little LY remained cell associated (<10 ng LY/mg protein).

To test whether LY uptake reflects binding to some serum component that might enter by adsorptive pinocytosis, we measured pinocytosis in the presence of various concentrations of fetal bovine serum. Cells incubated in serum-free Eagle's minimum essential medium plus LY exhibited the same biphasic uptake as cells in 5, 10, or 20% serum, with the same initial rate of uptake (Fig. 4). Steady-state rates of accumulation increased slightly as the serum concentration was increased (Fig. 4). To determine whether LY remained bound to cell protein after endocytosis, cells incubated for 3 h in Eagle's minimum essential medium with 1 mg/ml LY were washed, then lysed in Triton X-100, and that lysate was analyzed by Sephadex G-25 gel permeation chromatography at pH 5.0. 900 ng of LY were accumulated per milligram of cell protein, and of that, 8% was associated with high molecular weight material. Thus, since (*a*) the rate of fluid uptake was independent of the concentration of LY in the medium, (*b*) kinetics of accumulation were similar at different serum concentrations, and (*c*) there was little binding of LY to large molecules from cell lysates, we conclude that LY is a fluid marker.

LY Is Not Degraded

The apparent biphasic kinetics of uptake could reflect degradation of the LY. To examine this possibility, we measured

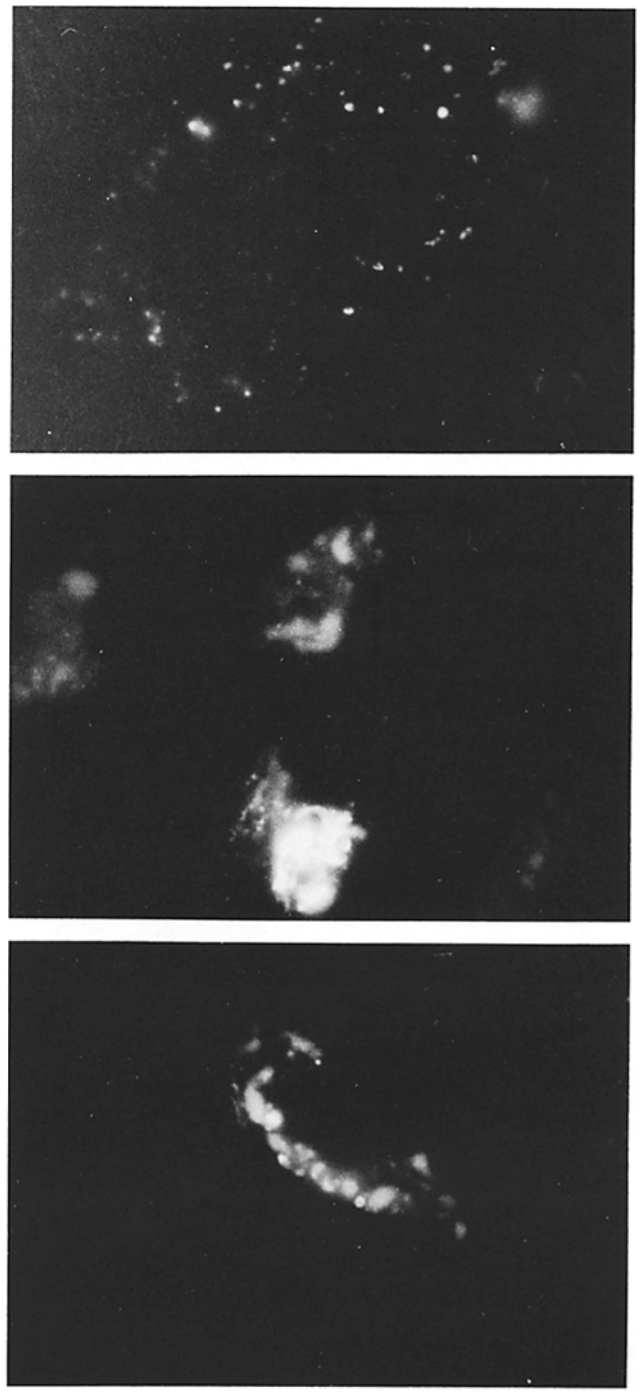

FIGURE 2 Fluorescence images of LY inside thio-macrophages (fixed cells). (*A*) 5-min pulse of 1.0 mg/ml LY. (*B*) 60-min pulse. (*C*) 60-min pulse + 120-min chase. × 100.

the stability of the probe's fluorescence following various periods of intracellular residence. Macrophages were incubated in LY, washed, and then reincubated in PBS + BSA for various periods of time (chase periods). The chase medium was collected, and the cells were rinsed with two more volumes of isotonic saline. The chase medium and rinses were pooled, and the fluorescence was measured. In addition, the cells attached to the dish were lysed in Triton X-100 as described, and the dish-bound fluorescence was measured. Although dish-bound LY declined with reincubation time, the total amount of LY (dish-bound plus chase medium)

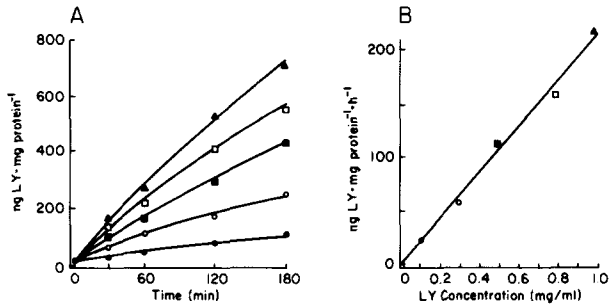

FIGURE 3 (*A*) Concentration dependence of uptake. Thio-macrophages were incubated in LY for the times indicated. ●, 0.1; ○, 0.3; ■, 0.5; □, 0.8; ▲, 1.0 mg/ml LY in M10F. (*B*) Linear rates of accumulation vs. LY concentration. Rates were calculated by linear regression of the uptake curve from 60 to 180 min. Plots of nanograms LY per milligram protein vs. LY concentration for each loading time also yielded straight lines that passed through the origin (not shown).

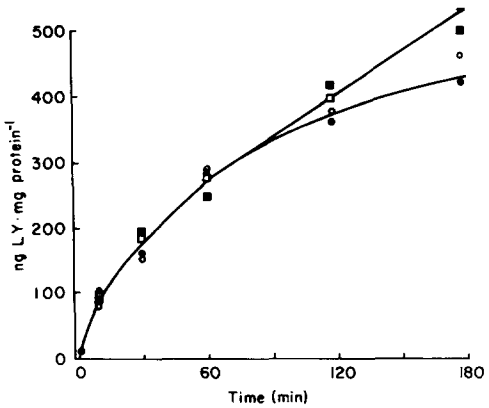

FIGURE 4 Effect of serum on LY uptake. Incubations contained 0.5 mg/ml LY and Eagle's minimum essential medium with various serum concentrations: ●, 0; ○, 5; ■, 10, and □, 20% serum.

remained unchanged. Thus, as measured by fluorescence yield, LY was not degraded over this 5-h chase period (Fig. 5).

Exocytosis

To measure exocytosis, cells were incubated for various times in LY-containing M10F, washed at 0°C, reincubated in LY-free M10F (plus 10 mM HEPES, pH 7.2) for various times, washed again, and lysed. Fluorescence of the lysate represents cell-associated LY. Exocytosis of LY exhibited biphasic kinetics: regardless of loading time (pulse), there was an immediate and rapid release upon return to 37°C in chase medium (Fig. 6). In addition to the rapid exocytosis there was a slower egress, which only became apparent in chases of 60 min or longer. The longer the loading time, the greater the amount of LY that left the cell during a given chase period. The release could be blocked by incubation at 0°C (data not shown).

When rates of efflux were measured after various loading times, we found that as the rate of accumulation declined toward steady state, the rate of exocytosis increased. The actual rate of pinocytosis could be estimated for any time point on the accumulation curve by adding the initial rate of efflux to the slope of the accumulation curve (Fig. 6). By such calculations it is evident that although initial accumulation was curvilinear, the rate of internalization (uptake plus efflux)

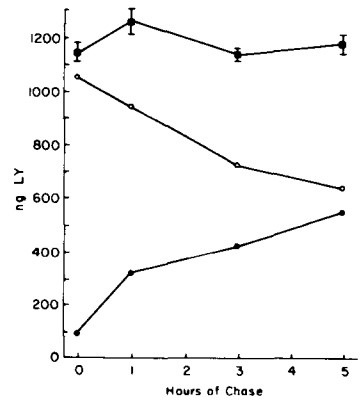

FIGURE 5 Efflux of LY from macrophages. Cells incubated for 12 h in 0.3 mg/ml LY in M10F were washed at 0°C, then reincubated for the indicated times in 0.50 ml PBS (+ 0.1 mg/ml BSA). At the end of that period, chase medium was collected and two rinses of cold PD (0.55 ml each) were added and then saved. Chase medium and rinses were pooled and their fluorescence was measured (●, medium). Cells remaining on the dish were lysed in Triton X-100 and this dish-associated fluorescence was measured (○, dish-bound). ■, total (medium fluorescence plus dish-bound fluorescence).

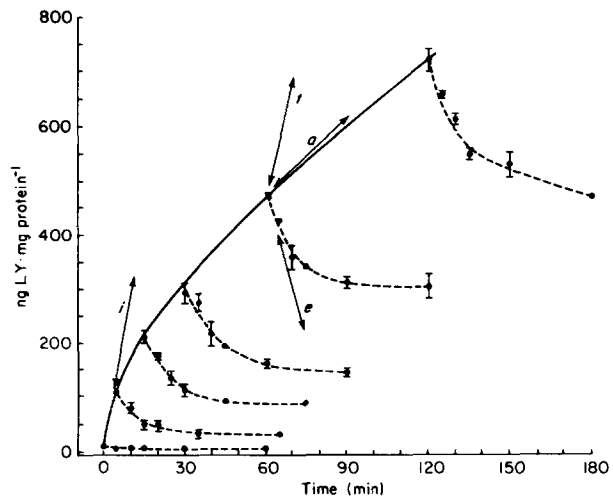

FIGURE 6 Pulse-chase measurements of LY uptake and release from thio-macrophages. Efflux was measured after various periods of incubation of thio-macrophages with 1.0 mg/ml LY. The initial rate of uptake was estimated as the tangent to the uptake curve at time 0 (line *i*). Subsequent rates of influx (e.g., line *t*) were calculated for various pulse times by addition of the rate of efflux (the tangent of the efflux curve at the start of each chase, line *e*) to the slope of the accumulation curve (line *a*). The solid line equals accumulation with no chase, dotted lines equal efflux, measured as amount remaining in cells. The values obtained by these methods are shown in Table I (control).

was nearly constant (Table I). When accumulation became linear, 80% of the internalized LY was returning to the medium (Table II, control). The cells accumulated 220 ng LY/mg protein per h (at 1.0 mg/ml LY), but internalized the dye at 1,200 ng/mg protein per h (Fig. 6). Assuming there are 5×10^6 cells/mg protein, this converts to an actual pinocytic rate of 240 fl/cell per h.

The amount of LY that left the cells during any chase period was directly proportional to the concentration of LY used during loading and thus reflects a fluid flow. Fig. 7*A*

TABLE I

*Effect of PMA on Rates of Fluid Influx and Efflux**

Condition	Pulse	Accumulation‡	Efflux‡	Total‡
		min		
Control	0	1,650	0	1,650
	5	820	470	1,300
	15	460	630	1,090
	30	360	700	1,060
	60	280	820	1,100
	120	220	980	1,200
PMA	0	3,200	0	3,200
	5	1,600	570	2,170
	15	950	1,100	2,150
	30	890	860	1,750
	60	930	1,060	1,990
	120	930	920	1,850

* Rates of uptake and efflux of 1 mg/ml LY in M10F. Calculations are from curves shown in Figs. 6 and 11.

‡ The rates reported for accumulation and efflux are the tangents to the respective curves (line *a* and line *e*) at the times indicated. (The values for *total* are from line *t*.) For instance, the rate of accumulation of LY at 15 min in control cells is the slope of the tangent to the solid line in Fig. 6 at the 15-min point. Similarly the rate of efflux at this time is the slope of the tangent to the dotted line in Fig. 6 at the 15-min point. We have expressed slopes as nanograms LY per milligram protein per hour.

shows the efflux from cells loaded 120 min with various concentrations of LY. The curves become superimposable when replotted as percentage of internalized LY remaining in cells (Fig. 7*B*).

The kinetics of uptake and of exocytosis we observe with LY are similar to those observed by Besterman et al. (4), using [^{14}C]sucrose as a probe. Interpreting their data by compartmental analysis, they described pinocytosis as the sum of two rates of uptake and two first-order rates of emptying. One kinetically defined compartment (compartment 1) is of relatively small volume, fills rapidly by pinocytosis, and rapidly returns a large portion of its contents to the medium by exocytosis. The remainder passes on to a second compartment, one which is apparently larger, fills more slowly, and returns fluid to the medium slowly. Besterman et al. (4) described a method of curve-peeling to analyze the two rates of efflux. When the percent of internalized probe remaining in cells is plotted on a log scale as in Fig. 7*B*, the slow rate of efflux is described by a straight line. The *y*-intercept of that line indicates the percentage of probe at the beginning of the chase that will exit at the slow rate (compartment 2). The remainder is the percentage of probe at the beginning of the chase that will exit rapidly (compartment 1). (If there were only one first-order rate of exocytosis, the *y*-intercept would be 100%.) Using these curve-peeling methods (4) we have calculated the approximate volume of the first compartment (48 fl/cell) and its half-time of emptying ($t_{1/2}$ = 6 min). We infer from our data that the first compartment has reached steady state by 60 min and that the subsequent linear accumulation of LY by the cells reflects only the net accumulation of the dye in the second compartment.

Effect of HRP on Pinocytosis of LY

In contrast to the kinetics observed with LY, initial accumulation of HRP by thio-macrophages is linear (reference 15 and Fig. 8*A*). A portion of the HRP uptake is adsorptive, as thio-macrophages contain mannose receptors at their surfaces and HRP contains mannose residues (13, 18). We used LY to examine the effects of HRP as a ligand on fluid influx and efflux. LY accumulation was immediately stimulated by the presence of HRP in the medium. Early LY accumulation was linear (Fig. 9). With longer incubations (≥3 h) the rate of accumulation decreased.

TABLE II

Effect of HRP on Rates of Fluid Influx and Efflux‡

Condition	Pulse	Accumulation*	Efflux*	Total*
		min		
Control	0	2,200	0	2,200
	30	560	1,100	1,660
	60	340	1,160	1,500
	120	230	1,020	1,250
	240	230	1,050	1,280
	360	230	1,330	1,560
HRP	0	3,200	0	3,200
	30	1,700	1,540	3,240
	60	800	1,620	2,420
	120	440	1,760	2,200

* Rates of uptake and efflux of 1 mg/ml LY in M10F. Calculations are from curves shown in Fig. 9. (*accumulation*, line *a*; *efflux*, line *e*; *total*, line *t*.) Rates were determined as shown in Fig. 6.

‡ Nanograms LY per milligram protein per hour.

To be sure that the observed stimulation was of fluid uptake and not due to LY adsorption to HRP, we examined the HRP-stimulated accumulation of LY in the presence or absence of 10 mg/ml mannan. Mannan competitively inhibits adsorptive uptake of HRP and reduces the rate of HRP accumulation by 40% (reference 21 and Fig. 8*A*). However, the presence of mannan had little effect on the HRP-stimulated accumulation of LY (Fig. 8*B*). We also examined directly the binding of LY to HRP by analyzing a mixture of LY and HRP by gel permeation chromatography. From an initial mix of 0.1 mg/ml LY and 1.0 mg/ml HRP, <1 mol of LY (457 mol wt) per 400 mol HRP (40,000 mol wt) separated with the high molecular weight fraction (HRP). This same mixture of LY and HRP enters cells by pinocytosis at a molar ratio of 8.5:1 (LY/HRP). Thus the binding of LY to HRP cannot account for the enhanced uptake of the dye in the presence of this protein.

Pulse-chase measurements of LY pinocytosis were carried out in the presence and absence of 1.0 mg/ml HRP (Fig. 9). Total pinocytic rate, calculated as in Fig. 6, was increased by HRP 1.67-fold, from 1,650 ± 350 to 2,760 ± 460 ng LY/mg protein per h. The rate of efflux was stimulated as well (1,090 ± 60 ng LY/mg protein per h increased 1.50-fold to 1,640 ± 90 ng LY/mg protein per h). Because the changes in rates of influx and efflux were disproportionate, the rate of accumulation increased more than did the rate of pinocytosis (Table II).

Stimulation of Pinocytosis by PMA

The tumor promoter PMA induces a two- to fourfold increase in accumulation of HRP by thio-macrophages. Congeners without tumor-promoting activity do not stimulate HRP uptake in these cells (11). This increase in HRP uptake reflects enhanced fluid-phase and not adsorptive pinocytosis (18). For this reason, we examined the effect of PMA on LY uptake. Fig. 10 shows that the rate of accumulation of LY was stimulated sevenfold immediately upon the addition of PMA or its water-soluble congener, phorbol dibutyrate. The accumulation rate remained high for several hours (data not

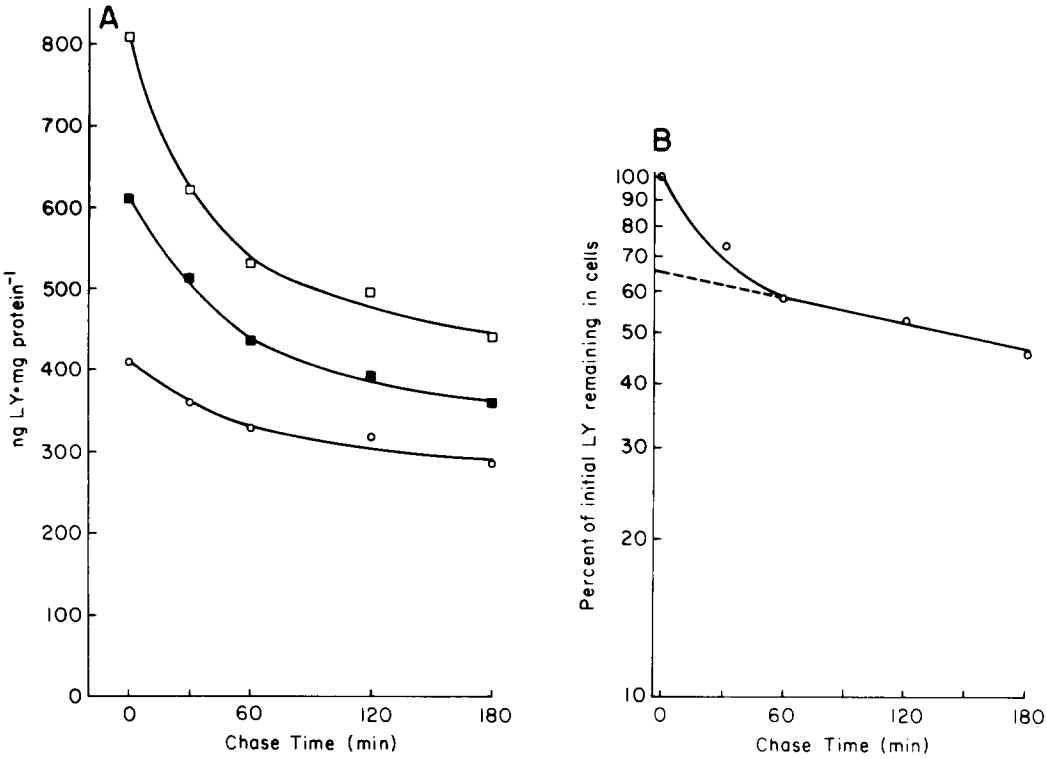

FIGURE 7 Efflux of LY from thio-macrophages. (*A*) Cells loaded for 120 min with either 0.3 (○), 0.6 (■), or 1.0 (□) mg/ml LY in M10F were washed in the cold and then reincubated in M10F for the times indicated. The amount of LY remaining in the cells is plotted vs. reincubation time. (*B*) Data for each of the three curves shown in *A* are replotted as the percentage of the initial LY (at chase time 0 min) remaining in the cells. For all three concentrations, the recalculated points for each chase time are superimposable and therefore describe the same line.

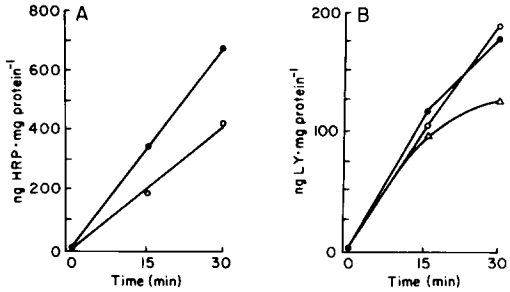

FIGURE 8 Pinocytosis of HRP and LY. (*A*) Accumulation of HRP (1.0 mg/ml) in the presence (○) or absence (●) of 10 mg/ml mannan. HRP accumulation appears linear and is inhibited 40% by mannan. The presence of LY in these incubations did not affect the accumulation of HRP (not shown). (*B*) Accumulation of LY. LY accumulation is stimulated the same extent with or without mannan in the medium. ○, 0.5 mg/ml LY, 1.0 mg/ml HRP, 10 mg/ml mannan; ●, LY + HRP; △, LY alone. One set of samples was used for both the HRP measurement and the LY measurement.

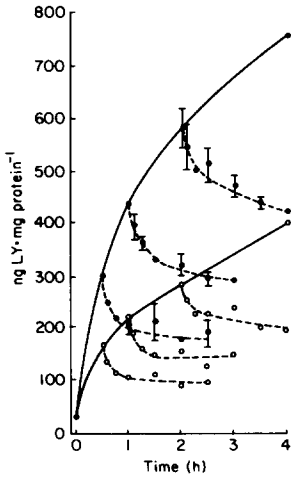

FIGURE 9 Pulse-chase measurement of LY pinocytosis in the presence of 1.0 mg/ml HRP. Methods were as described in Fig. 6. ○, accumulation control; ●, accumulation of LY with HRP. Dotted lines indicate efflux of LY. Rates are shown in Table II.

shown). When PMA was present throughout the experiment, the initial uptake kinetics more nearly approximated a straight line from the origin (Fig. 11). An initial curvilinear uptake phase was still measurable. Using the pulse-chase methods described above, we dissected this stimulation and found that after PMA treatment pinocytosis (the rate of accumulation plus the rate of exocytosis) was actually increased only 1.6 times. However, since the rate of exocytosis was only slightly increased, a greater percentage of the pinocytosed LY was retained, and the small increase in LY uptake was greatly amplified in the accumulation curve (Fig. 10 and Table I). Moreover, compartmental analysis of the efflux indicated that the amount that emptied rapidly (compartment 1) was unchanged with PMA treatment. Thus, it was the flow into the slow compartment (compartment 2) that was stimulated by PMA.

Cells examined live after pinocytosis of LY in PMA appeared more spread out on the substrate. The morphology of the fluorescent, LY-containing organelles was similar to that of unstimulated cells, but the long, tubular organelles were more prominent. They often extended from the vicinity of the nucleus (or cytocenter) toward the cell periphery as radial spokes (Fig. 12). The thinnest edges of the cell were dark. Upon addition of fixative (formaldehyde) the tubular organelles fragmented to form a pattern identical to that described for lysosomes by Phaire-Washington et al. (10). (The long tubules remained fluorescent after reincubation of cells in medium lacking LY, thus suggesting that these organelles were part of the slowly emptying compartment.)

DISCUSSION

Studies with various soluble probes (1, 9, 14) have defined pinocytosis as the linear accumulation of fluid and solutes and their irretrievable transit to the lysosomes. The great quantities of HRP pinocytosed by macrophages and fibro-

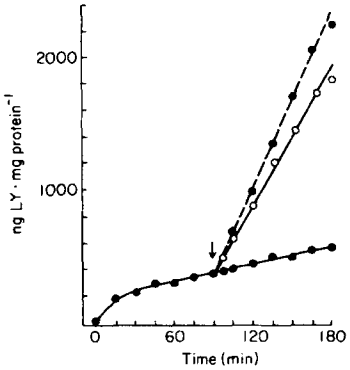

FIGURE 10 Phorbol esters immediately stimulate rates of LY accumulation. 10 ng/ml PMA (dotted line) or 500 ng/ml phorbol dibutyrate (○) in M10F was added at the time indicated by the arrow. Filled circles on the solid line indicate control rate of uptake (0.5 mg/ml LY in M10F).

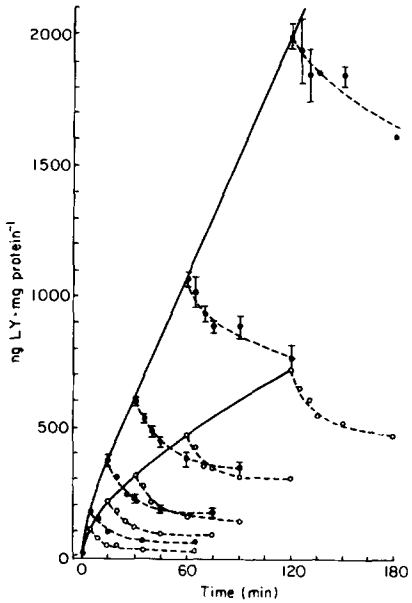

FIGURE 11 Pulse-chase measurement of LY pinocytosis in the presence (●) or absence (○) of 10 ng/ml PMA. Cells were preincubated 60 min in PMA before LY was added. Solid lines indicate accumulation with no chase, dotted lines indicate efflux. Values obtained are shown in Table I.

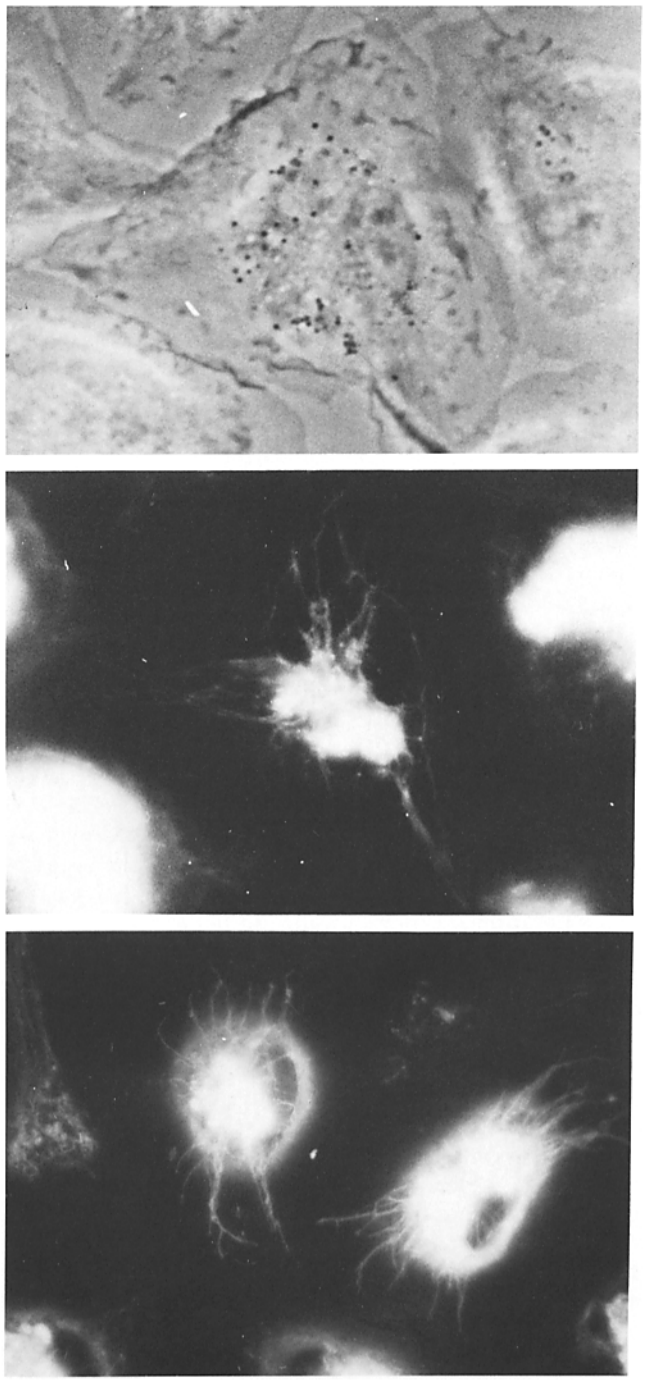

FIGURE 12 Living thio-macrophages incubated with 10 ng/ml PMA. (*A*) Phase-contrast image of 60-min loading of 1.0 mg/ml LY (+ PMA). (*B*) Same cell as in *A*, fluorescence image. (*C*) 60-min load of LY (+ PMA), then 120-min chase (+ PMA). Note the long, linear fluorescent organelles. × 100.

blasts prompted Steinman et al. (14) to suggest a return flow of membrane from pinosomes or lysosomes to the cell surface. However, if some fluid returns to the surface with recycling membrane, uptake kinetics should not be strictly linear from the origin. They should instead show rapid accumulation of marker at early time points, with subsequent slowing of accumulation as membrane recycling carries fluid and marker out of the cell.

Like Besterman et al. (4), we found the initial rate of pinocytosis to be curvilinear, rather than linear from the origin. We also found that internalized LY exits the cell at two rates, rapidly and slowly, both with first-order kinetics. From the conclusions of many published morphological studies of pinocytosis and from our fluorescence microscopic observations of these cells, we infer that the kinetically defined, rapidly filling and rapidly emptying compartment includes pinocytic vesicles, endosomes, and vesicles destined for the plasma membrane (16). By similar logic we identify the slowly filling, slowly emptying compartment as lysosomes. Accumulation of LY is curvilinear because the population of pinosomes (compartment 1) is filling and returning a significant proportion of the internalized medium to the surface rapidly, reaching steady state by 60 min. Meanwhile lysosomes (compartment 2) are filling and emptying more slowly, and account for the long, linear accumulation of LY.

The rate of probe accumulation by thio-macrophages is a poor indicator of the pinocytic rate. The linear rate of LY accumulation at steady state is five times less than the actual

rate of influx, largely because so much fluid is leaving the cells. Pinocytosis is best measured in the first few minutes of uptake or, more elaborately, by adding the rate of efflux to the rate of accumulation (Fig. 6). The rate of LY influx as measured by this more elaborate method appears slower than that measured at initial time. It may be that, because of the brief time required at the beginning of the chase period for the washed culture dishes to warm from 4° to 37°C, we are underestimating the initial rates of efflux. The finding that efflux remains constant at all dye concentrations (Fig. 7) suggests that LY is not affecting the uptake rate.

Morphological studies indicate that lysosomes receive fluid from pinosomes (1, 14), and there is kinetic evidence to support this concept (4). If compartment 2 received its fluid from compartment 1, then its rate of filling would depend on the efficiency of transfer from compartment 1. If the efficiency of transfer of solute from compartment 1 to 2 were increased, less fluid would leave the cell by rapid exocytosis and more would go to the slowly emptying compartment. The net effect would be that as the efficiency of transfer increased, the overall rate of accumulation would increase, and the uptake kinetics would more nearly describe a straight line from the origin, without requiring any change in the rate of internalization (pinocytosis). The nearly linear accumulation by PMA-stimulated thio-macrophages reflects such a change in efficiency of transfer, although in that case there is an increase in both pinocytic rate and flow to lysosomes.

Berlin and Oliver (2) used fluorescein isothiocyanate-dextran as a fluid-phase marker to measure pinocytosis in the murine macrophage-like cell line J774.2 and found accumulation to be linear with incubation time. Using LY, we also found linear uptake, but only after an initial period of more rapid and curvilinear accumulation. Pulse-chase experiments comparing J774.2 with thio-macrophages revealed that J774.2 cells have a greater rate of accumulation because a greater percentage of fluid coming into the cell is retained in lysosomes (i.e., enters compartment 2; data not shown.)

The compartmental analysis described by Besterman et al. (4) provides a useful approach to dissecting the component fluxes of pinocytic accumulation. However, we hesitate to use these methods to calculate pinocytic rate because of limitations we have found: (*a*) The method of curve-peeling, in which the rates of exocytosis are used to measure compartment dimensions, does not accurately estimate the size of compartment 1, but rather the size of that portion of the compartment that will exit rapidly. The portion of compartment 1 that moves on to compartment 2 is by such calculations included in compartment 2 from the beginning. If the efficiency of transfer from compartment 1 to 2 were to increase, the apparent size of compartment 1, as measured by curve peeling, would shrink, when in fact the real compartment dimensions may not change at all. (*b*) If compartment 2 is lysosomal, then it is likely that probe molecules are concentrated therein. Concentration of the probe precludes kinetic measurements of compartment size. This then prevents calculation of a rate constant for slow exocytosis and therefore also makes it difficult to quantify the actual rate of pinocytosis by compartmental analysis. Because of these difficulties, we have instead chosen to estimate pinocytic rate by adding the efflux rate to the rate of accumulation.

In thio-macrophages, HRP altered the kinetics of LY movement. Accumulation was increased and linear for several hours. Upon dissection of the process we found two explanations: (*a*) The total pinocytic rate, the sum of the accumulation curve and the initial efflux rate, was increased. Part of the HRP uptake in these cells is known to be receptor mediated (6, 18), and this process may increase fluid uptake by the formation of more pinosomes. (*b*) At early time points after HRP addition, a smaller percentage of the fluid entering the cells returned outside quickly. Receptor–ligand interactions involving HRP may direct more pinosomes on to lysosomes and fewer back out to the surface, thereby increasing the amount of probe retained and straightening the uptake curve. In a washout experiment, Steinman and Cohn (15) could detect no efflux of HRP from macrophages. We conducted a similar experiment, loading thio-macrophages for 3 h with 1 mg/ml HRP and 1 mg/ml LY, then measuring efflux as in Fig. 5. We found that while measurable quantities of LY appeared in the chase medium, no comparable efflux of HRP was detectable, even after accounting for the rate of enzyme degradation (data not shown). Most likely, as Steinman and Cohn (15) originally suggested, pinocytosed HRP goes directly to lysosomes and is degraded.

The stereological study of pinocytosis by Steinman et al. (14), using HRP as a morphological probe in mouse resident macrophages, should be reconsidered in light of the effects of HRP reported here. In a simple comparison, making general assumptions about pinosome size (surface area = 0.25 μm^2 and volume = 0.02 μm^3 [14]) and cell size (surface area = 1,000 μm^2 and volume = 450 μm^3 [10]), we find membrane and fluid turnover at steady state to be slightly faster than that measured by Steinman et al. (14). Thio-macrophages internalize the equivalent of their surface area every 20 min and their volume every 116 min. A rigorous comparison will require another stereological analysis with a nonadsorptive probe.

PMA dramatically alters pinocytosis by thio-macrophages. Accumulation was nearly linear and was four to seven times the steady-state rate of accumulation by unstimulated cells. An important finding of these studies is that the actual pinocytic rate was stimulated only 1.6 times by PMA. However, since the rate of exocytosis changed very little, the net effect was a dramatic increase in LY accumulation. This effect is shown diagramatically in Fig. 13. Further characterization of exocytosis showed that although LY was still leaving the cell at two rates, a greater percentage of it was in the slowly emptying compartment (compartment 2). It is likely, then,

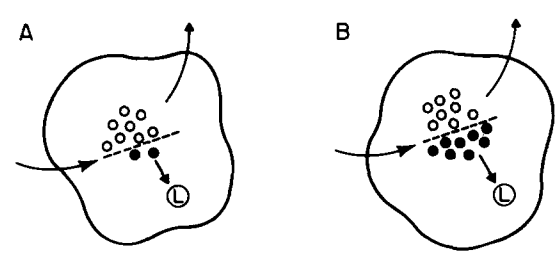

FIGURE 13 Accumulation of probe is a misleading indicator of pinocytic rate. Circles represent molecules of LY that enter the cell by pinocytosis. Open circles indicate LY that exits the cell. Filled circles indicate retained LY. (*A*) At steady state in the unstimulated cell, for every 10 molecules of LY that enter per unit time (pinocytosis), 8 leave. The net gain per unit time (accumulation) is two. (*B*) At steady state in the PMA-treated cell, the rate of pinocytosis has increased 1.6-fold to 16 molecules per unit time. Since the rate of exocytosis is unchanged (8 molecules per unit time), then 8 molecules remain in the cell and the rate of accumulation has increased fourfold over control rates.

that treatment with PMA either changed pinosome sorting such that the rapid recycling of fluid decreased and flow to lysosomes increased, or stimulated a qualitatively different type of pinocytosis which carried fluid straight to the lysosomes.

Kinetic arguments predict that in a compartment of constant dimensions that is both filling and emptying, the accumulation of LY should be curvilinear, eventually reaching some capacity. Steinman et al. (14) have shown that the lysosomal compartment dimensions remain constant. We and others have shown that accumulation of fluid-phase markers is linear. How can this apparent paradox be resolved? Besterman et al. (4) suggest that there might be a third compartment, receiving fluid from the second and returning none to the surface. When we looked at the fluorescence in cells we found compartment 2 to be made up of long tubules and many small organelles. If recycling lifespans were limited some organelles could eventually slow their rates of efflux and become permanent compartments. The morphologically described residual bodies (5) would be likely candidates for this endstage compartment. The long-term linearity of accumulation would thus result from a continual supply of new lysosomes, each recycling fluid and membrane for a few hours, then maturing to a less active, residual organelle with LY trapped inside. Alternatively, linear accumulation could simply reflect an increase in the dimensions of compartment 2. There is a limit to the linear accumulation. As mentioned above, accumulation slowed after 18 h in LY. This slowing may indicate that compartment 2, whose efflux is very slow ($t_{1/2}$ = 5–8 h), has reached capacity.

Actual fluid flow through cells may be difficult to measure. By the very nature of the investigation, cells must be exposed to an unusual encounter with large quantities of a nondegradable and impermeant molecule. Concentration of probe into lysosomes quite possibly distorts the normal flow into and through that compartment. Understanding the process therefore requires both kinetic and morphological studies, and LY is a good probe in both regards.

Our model of fluid-phase pinocytosis, based on the present studies and on the published work of others (12, 16), has flow into and through cells subject to regulation at several points. Most of what comes in goes back out. Internalized fluid either leaves quickly or is transferred to lysosomes. This must be a critical point for the regulation of traffic, as suggested by Besterman et al. (3). Ligation of mannose receptors by HRP or treatment with PMA appears to direct more flow to lysosomes. Once there, impermeant solutes not degraded by hydrolytic enzymes must be concentrated. Some return with membrane to the surface, possibly in small vesicles (19), while most remain within the lysosomes as the organelles mature.

The re-routing of fluid toward lysosomes brought about by ligands or phorbol esters could consequently affect cellular salt and water balance. The concentrative mechanisms of lysosomes may bring pinocytosed water and Na^+ into the cytosolic space, thus increasing both the flux of water through that space and the work of the Na^+/K^+ ATPases in the plasma membrane. Since PMA increases flow to (and through) lysosomes, it should in turn increase the ionic load on the cytoplasm.

The authors gratefully acknowledge the helpful suggestions of Drs. A. Aderem, J. Besterman, Z. Cohn, R. Steinman, and S. D. Wright.

This work was supported by Damon Runyon-Walter Winchell Cancer Fund Fellowship (DRG-588) and National Research Service Award No. 1 F32 AI 06880-01 CLN, both to J. Swanson and by U.S. Public Health Service grant No. AI 20516 to S. C. Silverstein.

Received for publication 17 August 1984, and in revised form 5 November 1984.

REFERENCES

1. Adams, C. J., K. M. Maurey, and B. Storrie. 1982. Exocytosis of pinocytic contents by Chinese Hamster ovary cells. *J. Cell Biol.* 93:632–637.
2. Berlin, R. D., and J. M. Oliver. 1980. Surface functions during mitosis. II. Quantitation of pinocytosis and kinetic characterization of the mitotic cycle with a new fluorescent technique. *J. Cell Biol.* 85:660–671.
3. Besterman, J. M., J. A. Airhart, R. B. Low, and D. E. Rannels. 1983. Pinocytosis and intracellular degradation of exogenous protein: modulation by amino acids. *J. Cell Biol.* 96:1586–1591.
4. Besterman, J. M., J. A. Airhart, R. C. Woodworth, and R. B. Low. 1981. Exocytosis of pinocytosed fluid in cultured cells: kinetic evidence for rapid turnover and compartmentation. *J. Cell Biol.* 91:716–727.
5. Daems, W. Th., E. Wisse, and P. Brederoo. 1972. Electron microscopy of the vacuolar apparatus. *In* Lysosomes: A Laboratory Handbook. J. T. Dingle, editor. Elsevier/North-Holland Biomedical Press, Amsterdam. 150–199.
6. Kaplan, J., and M. Nielsen. 1978. Pinocytic activity of rabbit alveolar macrophages *in vitro*. *J. Reticuloendothel. Soc.* 24:673–685.
7. Lowry, O. A., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
8. Miller, D. K., E. Griffiths, J. Lenard, and R. A. Firestone. 1983. Cell killing by lysosomotropic detergents. *J. Cell Biol.* 97:1841–1851.
9. Ose, L., T. Ose, R. Reinertsen, and T. Berg. 1980. Fluid endocytosis in isolated rat parenchymal and non-parenchymal liver cells. *Exp. Cell Res.* 126:109–119.
10. Phaire-Washington, L., S. C. Silverstein, and E. Wang. 1980. Phorbol myristate acetate stimulates microtubule and 10-nm filament extension and lysosome redistribution in mouse macrophages. *J. Cell Biol.* 86:641–655.
11. Phaire-Washington, L., E. Wang, and S. C. Silverstein. 1980. Phorbol myristate acetate stimulates pinocytosis and membrane spreading in mouse peritoneal macrophages. *J. Cell Biol.* 86:634–640.
12. Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. *Annu. Rev. Biochem.* 46:669–721.
13. Stahl, P. D., J. S. Rodman, J. M. Miller, and P. H. Schlessinger. 1978. Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. *Proc. Natl. Acad. Sci. USA.* 75:1399–1403.
14. Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. A stereological analysis. *J. Cell Biol.* 68:665–687.
15. Steinman, R. M., and Z. A. Cohn. 1972. The uptake, distribution and fate of soluble horseradish peroxidase in mouse peritoneal macrophages in vitro. *J. Cell Biol.* 55:186–204.
16. Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* 96:1–27.
17. Stewart, W. W. 1978. Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. *Cell.* 14:741–759.
18. Sung, S.-S. J., R. S. Nelson, and S. C. Silverstein. 1983. The role of the mannose/N-acetylglucosamine receptor in the pinocytosis of horseradish peroxidase by mouse peritoneal macrophages. *J. Cell. Physiol.* 116:21–25.
19. vanDeurs, B., and K. Nilausen. 1982. Pinocytosis in mouse L-fibroblasts: ultrastructural evidence for a direct membrane shuttle between the plasma membrane and the lysosomal compartment. *J. Cell Biol.* 94:279–286.