

Differential and Sequential Delivery of Fluorescent Lysosomal Probes into Phagosomes in Mouse Peritoneal Macrophages

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Abstract. It has previously been inferred that the fusion of a macrophage secondary lysosome with a phagosome delivers the entire lysosomal contents uniformly to the phagosome. We found, however, that different fluorescent lysosomal probes can enter phagosomes at remarkably different rates, even though they are initially sequestered together in the same organelles. Thus, sulforhodamine is almost exclusively

delivered to yeast-containing phagosomes within 2 h of phagocytosis. But fluoresceinated, high molecular weight dextran accumulates in the same phagosomes only over a period of ~24 h. We postulate that the delivery of lysosomal contents may involve an intermittent and incremental process in which individual components can be selectively and sequentially transferred.

ALTHOUGH it is demonstrable that the contents of lysosomes are transferred into phagosomes shortly after phagocytosis, the exact mechanism is not clear. It is commonly believed that the delivery occurs via phagosome-lysosome fusion, during which the lysosomal membrane becomes incorporated into the phagosomal membrane, thus allowing the entire contents of lysosomes to be transferred rapidly into the phagosomes (3, 7, 10, 16). Although earlier light and electron microscopic observations are consistent with such a model, other possibilities cannot be ruled out. From more recent studies, it is also clear that contents of intracellular membrane-bound organelles can be transferred in different ways. For example, Willingham and Yamada reported that pinosomes interact with lysosomes through repeated collision and segmentation, which they termed "piranhalysis" (23). In our light and electron microscopic studies of lysosome-phagosome fusion (14, 15), we have also encountered observations that cannot be easily explained by an all-or-none transferring process.

One possible approach for analyzing the transfer of molecules from lysosomes to phagosomes is to compare the behavior of probes of different properties. Specifically, if the transfer occurs through complete fusion, all probes should be delivered at an identical rate. Conversely, different rates might be detected if other mechanisms are involved. In the present study, we labeled secondary lysosomes with a mixture of sulforhodamine 101 (SR) and high molecular weight

fluorescein-dextran (FD). We found that even though they are initially sequestered together in the same lysosomes, their rates of entering phagosomes are very different. These observations lead us to propose alternative mechanisms for lysosome-phagosome interactions.

Materials and Methods

Cell Culture and Fluorescent Labeling

Stock solutions of fluorescent probes were prepared by dissolving the material in Dulbecco's PBS/FD (average molecular weight, 40,000; Sigma Chemical Co., St. Louis, MO) and rhodamine-dextran (average molecular weight, 70,000; Sigma Chemical Co.) at a concentration of 40 mg/ml; SR (Molecular Probes, Inc., Junction City, OR) at 2.5 mg/ml; Lucifer Yellow (LY; Molecular Probes, Inc.) at 5 mg/ml; and lissamine rhodamine B (LR, prepared by alkaline hydrolysis of the sulfonyl chloride purchased from Eastman Kodak, Rochester, NY), at a concentration of 40 mg/ml. Fluorescent dextrans were sometimes dialyzed against PBS to remove toxic contaminants. The RD stock solution was further purified by gel filtration in a Sephadex G-150 column (2.5 × 50 cm) to remove low molecular weight components. The stock solutions were filter-sterilized and stored in the dark at 4°C.

Unstimulated mouse peritoneal macrophages were recovered from young Swiss-Webster mice by lavage, and cultured in 6% CO₂ on special observation dishes (22) or on "flying" coverslips in Leighton tubes using DME (Gibco, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (HyClone Laboratories, Inc., Logan UT). The medium was changed every other day. After 3–4 d in culture, cells were fluorescently labeled by adding aliquots of stock solutions of fluorescent probes and incubating for 3–4 d. The final concentration was 2–3 mg/ml for FD, 0.7 mg/ml for RD, 35–70 µg/ml for SR when mixed with FD and 125 µg/ml when mixed with LY, 250 µg/ml for LY, and 300 µg/ml for LR. No cellular damage was detected after labeling. Labeled cells were subsequently washed with Hanks' balanced salt solution and incubated for up to 15 h in the culture medium. The medium was replaced immediately before observation or induction of phagocytosis.

1. *Abbreviations used in this paper:* FD, fluorescein-dextran; LR, lissamine rhodamine B; LY, Lucifer Yellow; RD, rhodamine-dextran; SR, sulforhodamine 101.

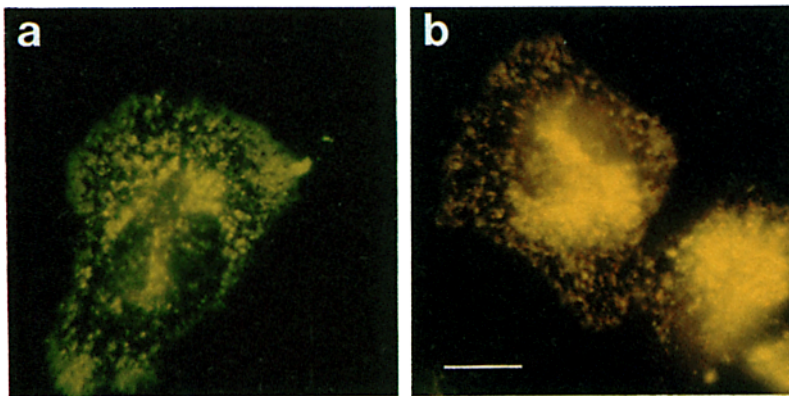


Figure 1. Sequestration of FD and SR in the same cytoplasmic compartment (lysosomes). Macrophage monolayers were exposed to FD alone or exposed simultaneously to SR and FD as described in the text and examined after a prolonged chase. With excitation at 485 nm and a 520-nm barrier, cells exposed only to FD (*a*) show the yellowish-green fluorescence characteristic of fluorescein. But with the same filter combinations, cells that had accumulated both fluors (*b*) appear orange-yellow, indicating that the dyes are colocalized in the same organelles. Some individual lysosomes are resolved. Bar, 10 μ m.

Induction of Phagocytosis

Baker's yeast (*Saccharomyces cerevisiae*) killed by boiling were washed and opsonized at 37°C for 30 min with 1:1 fresh mouse serum and DME at a concentration of 2×10^8 cells/ml. The yeast cells were washed, suspended in DME to a concentration of 10^6 cells/ml, and 1.5 ml of the suspension was added to each macrophage monolayer in dishes (or 0.75 ml/Leighton tube). After 45 min, the medium and unattached yeast cells were discarded and replaced with fresh complete medium.

Microscopy

For prolonged observations, macrophages on special observation dishes were maintained at 35°C in humidified CO₂ air on the stage of a Zeiss IM35 inverted microscope (22). A 100W quartz-halogen lamp and an epilluminator were used together with the Zeiss 487715 filter set (546 nm excitation, 590 nm barrier) for specific observation of SR or LR fluorescence, and the Zeiss 487716 filter set (485 nm excitation, 520 nm barrier) for simultaneous observation of fluorescein and rhodamine fluorescence. In some experiments, an extra barrier filter (560 nm; Spectra Film, Winchester, MA) was used in combination with the 487716 filter set for observing FD uncontaminated by fluorescence from LR or SR.

For short-term observations involving the comparison of SR and LY, coverslips with macrophages were inverted and mounted on a slide in DME and examined with a Zeiss photomicroscope and darkfield illumination. Excitation and barrier filters with characteristics similar to those of the 487715 set were used for SR. For LY, we used a combination of BG38 and BG12 filters for excitation and a 530-nm barrier filter. Photography was performed using Kodak VR 1000 film with an exposure time between 4 and 60 s.

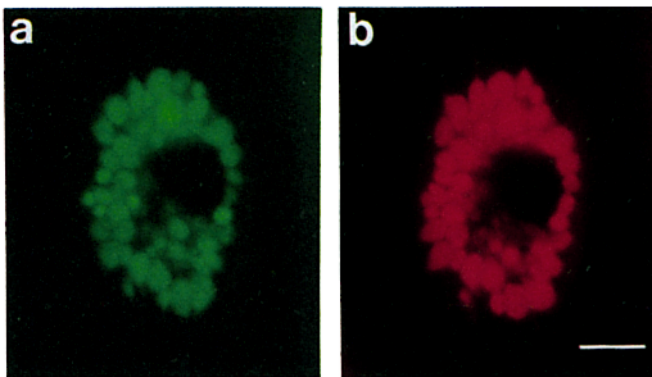


Figure 2. Colocalization of SR and FD in macrophage lysosomes. Cells were exposed for 3 d to the two fluors as described, thoroughly washed, chased for 2 h, and exposed to chloroquine diphosphate (15 μ g/ml) for 1 h. Photography was performed with filter sets selective for fluorescein (*a*) or rhodamine (*b*). The two fluors are found together in nearly all of the swollen lysosomes. Bar, 10 μ m.

Results

Sequestration of Probes

We used small, sulfonated, highly charged fluorescent molecules, including LR, SR, and LY, as one class of lysosomal probes. Unlike the lysosomotropic weak bases (1, 8, 13) these charged molecules do not directly permeate cellular membranes. Instead, they are most likely internalized by pinocytosis (2, 6, 8, 17, 21), delivered to secondary lysosomes, and are largely retained there because of their highly ionic nature (8). After uptake by macrophages, all three probes showed the same discrete punctate-to-vacuolar appearance characteristic of secondary lysosomes, and exhibited saltatory motions that were quite dramatic when viewed with time-lapse video recordings.

FD and RD were used as the second class of probe. Their cellular distribution appeared similar to that of the lower molecular weight markers. In addition, when cells had been exposed to a combination of FD and SR, most secondary lysosomes contained both probes. This was demonstrated using two approaches. First, lysosomes in these cells were orange-

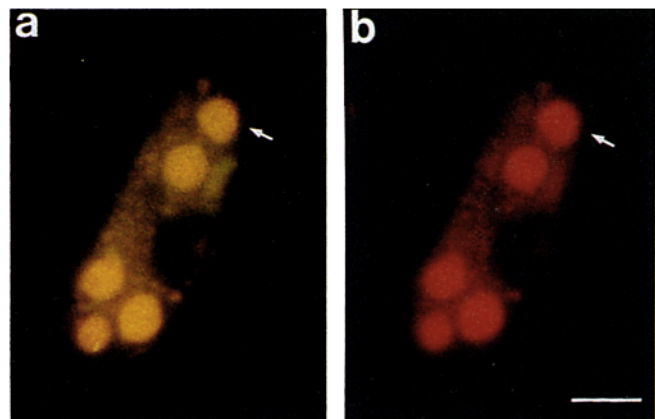


Figure 3. Simultaneous delivery of sulfonated ionic probes to yeast phagosomes. Cells were labeled for 4 d with both SR (125 μ g/ml) and LY (250 μ g/ml), washed and chased for 5 h, and given opsonized heat-killed yeast to phagocytose. After 2.5 h both dyes had been delivered to phagosomes (arrows). (*a*) Cells examined for LY; and (*b*) for SR. The cells appeared nearly degranulated with respect to both fluors. Some individual lysosomes are resolved, showing coexistence of both probes. Bar, 5 μ m.

yellow in color when observed with the 485-nm excitation and the 520-nm barrier filter, which allows fluorescence of both FD and SR to be detected (Fig. 1 *b*). This color was clearly different from that of lysosomes in cells loaded with FD alone (Fig. 1 *a*). Within individual cells, the color appeared quite uniform save for minor subpopulations of principally red or green lysosomes. In the second approach, cells

loaded with both FD and SR were treated with chloroquine diphosphate in DME (15 $\mu\text{g/ml}$) for 1 h to swell the lysosomes (8, 19), so as to achieve improved definition and to confirm more rigorously that the labeled organelles were secondary lysosomes (see references 19 and 24). They were then photographed with filter sets that selected specifically for either fluorescein or rhodamine fluorescence. As shown

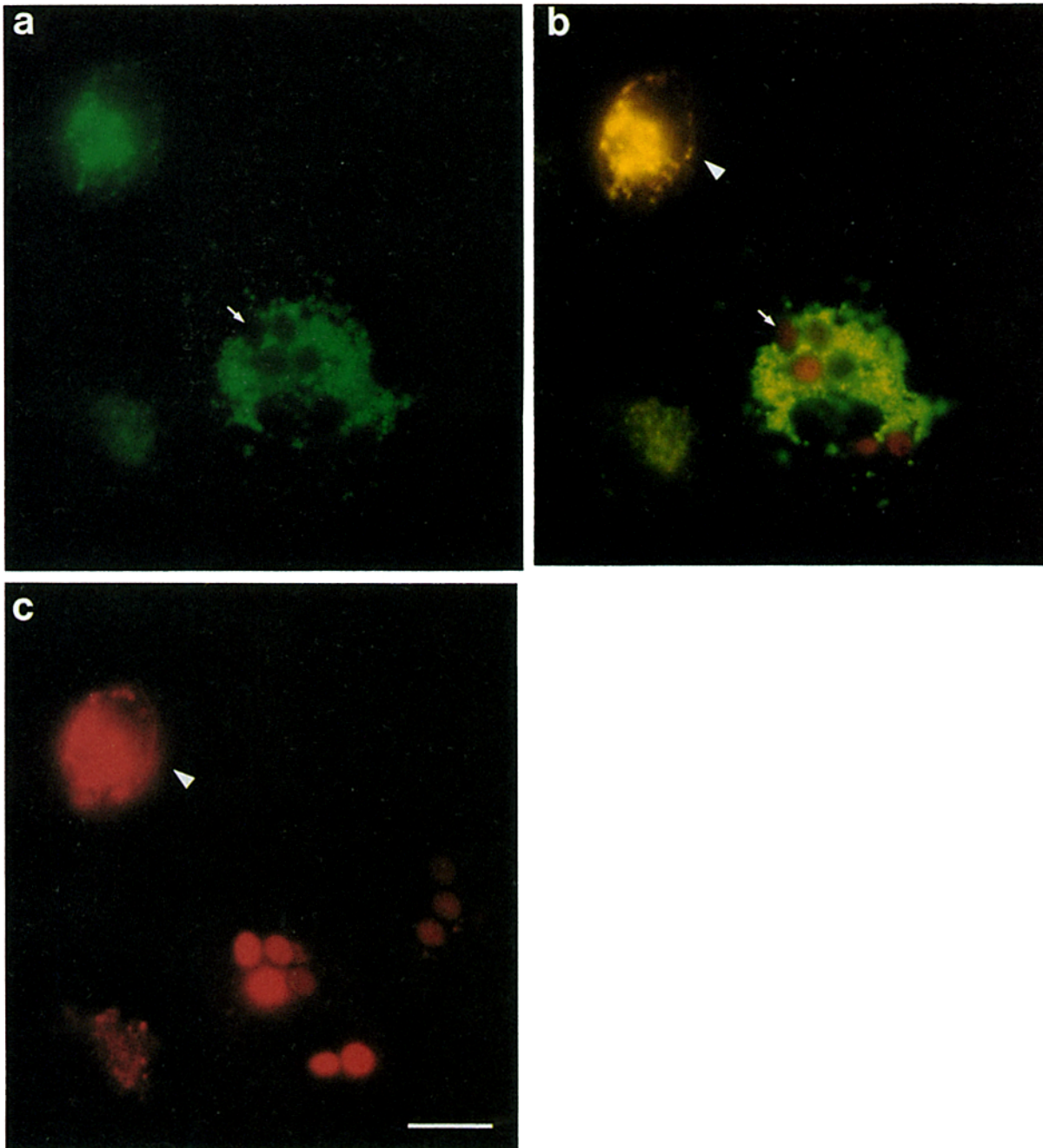


Figure 4. Preferential delivery of SR to yeast phagosomes in cells doubly labeled with SR and FD. After a 45-min phagocytic pulse, delivery of only SR is detectable after 2–3 h. (*a*) Cells examined for fluorescein (485 nm excitation and the 520/560 barrier combination) show a uniform background of greenish lysosomes. Phagosomes are seen as dark spaces (*arrow*) against the lysosomal background. (*b*) With the same excitation but with the 560 barrier filter removed, the red component of SR emission is recognizable and now several yeast phagosomes (*arrow*) give evidence of SR influx. (*c*) The same field examined with the “rhodamine set” (text) shows nine SR-containing yeast phagosomes of various fluorescence intensities. The central cell with six phagosomes appears degranulated, while the hungry cell that has not phagocytosed shows the full complement of both fluors (*arrowheads*). Bar, 10 μm .

in Fig. 2, *a* and *b*, the two probes were colocalized in more than 95% of the resolved lysosomes.

Delivery of Probes into Phagosomes

In order to follow the delivery of lysosomal contents into phagosomes, we first fluorescently labeled cells with various probes either alone or in combination and then fed them with heat-killed yeasts. For all the small, sulfonated probes, delivery occurred in a similar fashion and rate, as described earlier for LR (11, 13). When combinations of LY with either LR or SR were used, the yellow and red probes were delivered simultaneously to yeast-containing phagosomes after phagocytosis (Fig. 3, *a* and *b*).

The picture was considerably different, however, with cells that had been exposed to a combination of FD and SR. 2 h after the phagocytotic pulse, phagosomes contained little FD and appeared as dark spaces against a uniform greenish background of lysosomes when fluorescein fluorescence was selectively observed (Fig. 4 *a*). When fluorescence of fluorescein and rhodamine was observed simultaneously, phagosomes showed variable intensities of red color (Fig. 4 *b*, arrows). At this time (2 h), the lysosomes, on the other hand, were a yellowish-green color and thus similar to lysosomes labeled with FD alone (Fig. 1 *a*), indicating that the SR contents had been severely depleted. When the macrophages were examined for SR alone (Fig. 4 *c*), the cells appeared "degranulated", i.e., they contained few red fluorescing lysosomes but did contain brightly fluorescent red phagosomes. However, because of the widespread persistence of lysosomes as revealed by the 485-nm excitation (Fig. 4, *a* and *b*) it is erroneous to conclude that the macrophages have been degranulated. Exposure of these cells to chloroquine to raise intralysosomal (8, 18, 19, 24), and presumably intraphagosomal, pH did not alter the appearance of these phagosomes (see Discussion).

Fig. 4 also shows a "hungry" cell that has not ingested yeast (arrowhead). Here both probes are present in the lysosomes, which appeared orange-yellow in color when fluorescence of fluorescein and rhodamine were observed simultaneously (Fig. 4 *b*). The color was similar to that of lysosomes in cells before phagocytosis (cf. Fig. 1 *b*), indicating that little SR was lost spontaneously from the cell over the 2–3 h of incubation.

Sequential Delivery of SR and FD into Phagosomes

With cells loaded with either FD alone or with FD and SR in combination, delivery of FD became apparent only ~4–5 h after phagocytosis and increased steadily for 24 h (Fig. 5). RD, purified as described in Materials and Methods, behaved in a similar fashion (not shown). We therefore compared the time course of transfer of FD and SR into phagosomes for up to 26 h after phagocytosis, using cells labeled with a combination of the two probes and filter sets specific for fluorescein or rhodamine.

As shown in Table I, at early stages, essentially only SR was delivered to phagosomes (only 6% of 54 red phagosomes scored also contained detectable FD). At 4 h, the delivery of FD was only slightly greater (9%). But during the succeeding 22 h, the frequency and intensity of FD labeling of phagosomes grew, while that of SR labeling steadily declined after peaking between 4 and 12 h. At 24 h the num-

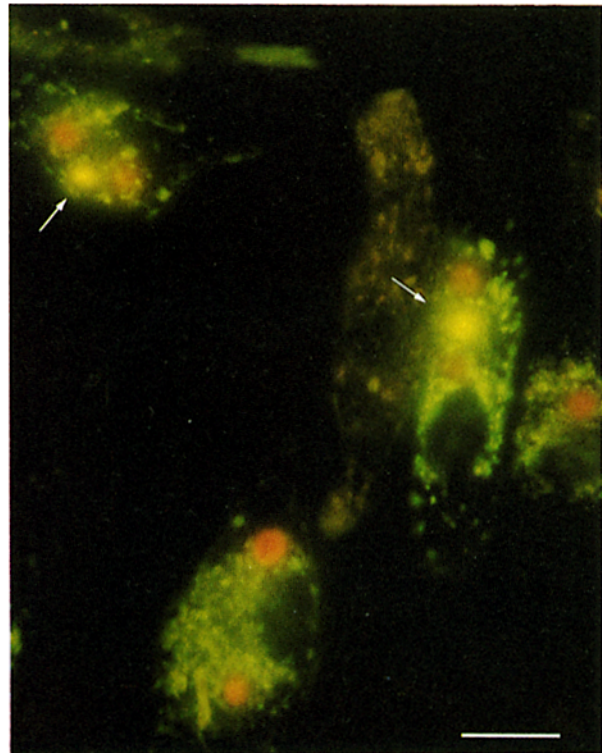


Figure 5. A spectrum of phagosomes to which various quantities of SR and FD have been delivered ~4–5 h postphagocytosis. Cells were prelabeled with both fluors as described for Fig. 3. In this field 2 out of 10 phagosomes give evidence of FD delivery (arrows). Bar, 10 μ m.

bers of phagosomes scored for SR and for FD were essentially the same. However, a few phagosomes contained only FD. These may represent yeast that were ingested quite late in the experiment, after most of the lysosomal SR had been transferred into earlier phagosomes. The overall intensity of SR also became weaker, possibly due to the release of SR into the medium, similar to the release of other low molecular weight products during the digestion of labeled macromolecular substrates (4, 5, 11, 19, 20, 24).

Discussion

Because of the extended chase periods used in our experiments, it is unlikely that the pinosomal compartment, which participates in the original labeling of the lysosomes, accounts for a significant proportion of the fluorescent organelles observed before phagocytosis. The half-life of components in pinosomes is very short (~5 min); thus any probe that remains associated with the cell after even an hour of chase is most likely localized within lysosomes (21). As evidence of the actual lysosomal accumulation of FD, Ohkuma and Poole (18) as well as Geisow et al. (9) have demonstrated, using a density gradient, that this probe becomes colocalized with lysosomal enzymes after pulse labeling and chase. By similar techniques, Miller et al. demonstrated the lysosomal sequestration of LY (17; see also 21). Our observations further suggest that LY and SR share the same intracellular domains; and that FD and SR are colocalized in secondary lysosomes that appeared as swollen organelles after uptake

Table I. Time Course of SR and FD Delivery to Yeast Phagosomes

Time after phagocytosis h	Labeled yeast phagosomes		
	SR	FD*	FD/SR ratio
2	54	3	0.055
4	236	22	0.093
13	125	65	0.520
24	184	166	0.902

* Although some phagosomes, usually later in the experiment, contained FD alone (see text) most of them contained both fluors. Cells labeled with the two probes, as described earlier, were allowed to phagocytose opsonized, heat-killed yeast and were incubated for 24 h. At the times indicated SR-containing red phagosomes and FD-containing green phagosomes were counted. Ratios of the two numbers were then calculated.

of chloroquine (Fig. 2). Therefore, our point of departure is that the probes used here reside together within secondary lysosomes at the time of phagocytosis.

However, despite the initial colocalization of SR and FD, only SR appeared rapidly in the phagosomes after the ingestion of yeast. Delivery of the polymeric FD became recognizable only after ~4–5 h, and then progressed slowly to high levels during a 24-h period. Similar behavior was seen in cells labeled with RD. In contrast, the various small, sulfonated probes were all delivered at an equally rapid rate into phagosomes.

Before attempting an interpretation of this interesting observation, several possible artifacts must be considered. First, it might be argued that the initial absence of FD fluorescence in phagosomes may be due to a low pH within these organelles. However, this is unsupported by the relatively rapid appearance of FD in yeast-containing phagosomes when macrophages are lightly labeled with this probe (13); by the brilliance of fluoresceinated yeasts in phagosomes (11); and, in the present experiment, by the inability of chloroquine to alter the appearance of the phagosomes. Second, it is also unlikely that the highly ionic, low molecular weight markers used in this study can reach the phagosomes by direct penetration through the lysosomal and phagosomal membranes. Unlike acridine orange (1), these probes are unable to permeate the cellular membranes and accumulate in lysosomes only after prolonged exposure, presumably through endocytosis or pinocytosis (17). Furthermore, once localized in lysosomes, they cannot redistribute to extracellular strong base or strong acid ion-exchange resins (our unpublished observation), whereas acridine orange is rapidly depleted from lysosomes by Dowex 50 (11–13). Therefore, although there may be unidentified pathways mediating the direct sequestration of small lysosomal molecules into phagosomes, it is difficult to explain the present results as being due to any known artifacts.

If the behavior of the probes observed here indeed reflects the actual interactions between lysosomes and phagosomes, what mechanisms can be invoked, then, to account for the differential and sequential delivery process? As one possibility, secondary lysosomes might fuse transiently and repeatedly with phagosomes. During each fusion, only a partial transfer of components takes place. Alternatively, lysosomes and phagosomes may also interact through a hypothetical or-

ganelle which shuttles between the two components and gradually delivers lysosomal contents into phagosomes. In both cases, the rate of delivery is likely to vary from component to component. For example, if the delivery involves passive diffusion of molecules into phagosomes, the rate would be determined by the mobility, and factors such as molecular weight, rheological properties, and membrane association may play an important role. It is also possible that specific channels, which selectively facilitate the delivery of certain molecules, may be involved. In this case, the rate may be affected by a wide spectrum of properties including size, hydrophobicity, and electric charge, in addition to mobility within lysosomes.

The low mobility of FD in lysosomes is indeed suggested by measurements of fluorescence polarization. According to Geisow et al. (9), the high degree of polarization of lysosomal FD is comparable to that measured in 90% glycerol, so that the motion of the molecule in the intralysosomal environment is likely to be highly restricted. The physical properties of FD that contribute to the reduced mobility are uncertain at present. However, since RD behaves similarly to FD, it is unlikely that the mobility is determined by the fluorophore. One possibility is that sufficiently concentrated dextrans may exist within lysosomes as gelatinous hydrosols (14, 15), which severely restrain the motional freedom of individual molecules.

The differential and sequential delivery of the lysosomal contents in macrophages appears to be quite different from that documented by Hirsch in polymorphonuclear leukocytes (3, 16). Using phase-contrast cinematography, he detected an explosive disappearance of cytoplasmic granules, which, in these cells, are primary lysosomes (6), as they interacted with the phagosomes. Hirsch's observations imply an abrupt, total delivery of granule contents into the phagosomes. However, to our knowledge, similar behavior in the fusion of secondary lysosomes with phagosomes in macrophages has not been described. It may now be fruitful to apply high resolution light microscopy and time-lapse recording to directly visualize the interactions between secondary lysosomes and phagosomes in macrophages.

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