

Delivery of Ligands from Sorting Endosomes to Late Endosomes Occurs by Maturation of Sorting Endosomes

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Abstract. After endocytosis, lysosomally targeted ligands pass through a series of endosomal compartments. The endocytic apparatus that accomplishes this passage may be considered to take one of two forms: (a) a system in which lysosomally targeted ligands pass through preexisting, long-lived early sorting endosomes and are then selectively transported to long-lived late endosomes in carrier vesicles, or (b) a system in which lysosomally targeted ligands are delivered to early sorting endosomes which themselves mature into late endosomes. We have previously shown that sorting endosomes in CHO cells fuse with newly formed endocytic vesicles (Dunn, K. W., T. E. McGraw, and F. R. Maxfield. 1989. *J. Cell Biol.* 109:3303–3314) and that previously endocytosed ligands lose their accessibility to fusion with a half-time of ~ 8 min (Salzman, N. H., and F. R. Maxfield. 1989. *J. Cell Biol.* 109:2097–2104). Here we have studied the properties of individual endosomes by digital image analysis to distinguish between the two mechanisms for entry of ligands into late endosomes. We incubated TRVb-1 cells (derived from CHO cells) with diO-LDL followed, after a variable chase, by diI-LDL, and measured the diO content of diI-containing endosomes. As the chase period was lengthened, an increasing percentage of the endosomes containing diO-LDL from the initial incubation had no detectable

diI-LDL from the second incubation, but those endosomes that contained both probes showed no decrease in the amount of diO-LDL per endosomes. These results indicate that (a) a pulse of fluorescent LDL is retained by individual sorting endosomes, and (b) with time sorting endosomes lose the ability to fuse with primary endocytic vesicles. These data are inconsistent with a preexisting compartment model which predicts that the concentration of ligand in sorting endosomes will decline during a chase interval, but that the ability of the stable sorting endosome to receive newly endocytosed ligands will remain high. These data are consistent with a maturation mechanism in which the sorting endosome retains and accumulates lysosomally directed ligands until it loses its ability to fuse with newly formed endocytic vesicles and matures into a late endosome. We also find that, as expected according to the maturation model, new sorting endosomes are increasingly labeled during the chase period indicating that new sorting endosomes are continuously formed to replace those that have matured into late endosomes. Also consistent with the maturation model, we observe no increase in the number of compartments labeled with a pulse of diO-LDL as would be expected if a pulse of ligand first labeled sorting endosomes and later labeled both sorting and late endosomes.

THE membrane economy of the cell depends upon a trafficking system that guarantees accurate routing of proteins on both the biosynthetic and endocytic pathways. Receptor-mediated endocytosis depends upon mechanisms that guarantee proper routing of internalized ligands and receptors. A variety of different ligands and receptors are internalized into common endosomes (Maxfield et al., 1978; Pastan and Willingham, 1983; Ward et al., 1989), from which they are directed to a variety of destinations. For example, low density lipoprotein (LDL)¹ is degraded in lysosomes, while its receptor is returned to the plasma membrane (Goldstein et al., 1979). EGF is degraded in lyso-

somes as is its receptor (Carpenter and Cohen, 1979). Transferrin (Tf) is returned to the plasma membrane along with its receptor (Dautry-Varsat et al., 1983; Klausner et al., 1983). Polymeric immunoglobulin A is internalized on the basolateral plasma membrane of certain epithelial cells, proteolytically cleaved, and the resultant fragment is secreted at the apical domain (reviewed in Mostov and Simister, 1985).

In the lysosomal pathway of endocytosis, ligands bind to receptors at the cell surface and are rapidly internalized into small endocytic vesicles. Ligands and their receptors are next found in somewhat larger tubulo-vesicular sorting endosomes (Geuze et al., 1983; Griffiths et al., 1989). At this point recycling receptors are sorted from lysosomally targeted ligands in an iterative process in which the recycling components are removed in recycling vesicles which repeat-

1. *Abbreviations used in this paper:* LDL, low density lipoprotein; Tf, transferrin.

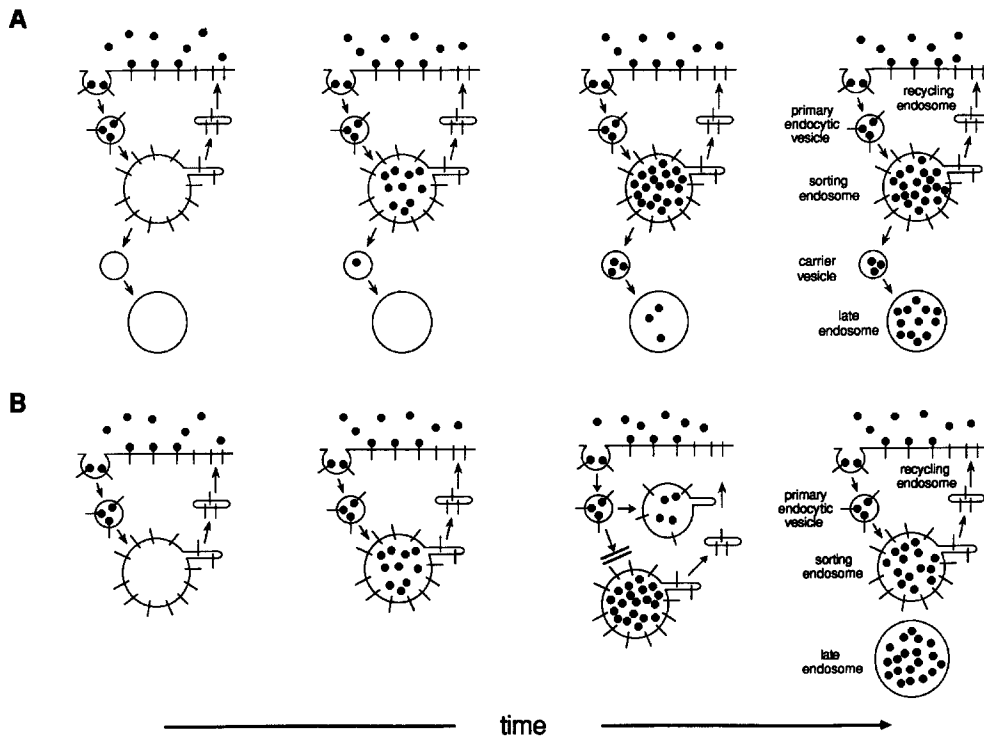


Figure 1. Schematic diagrams of the preexisting compartment model of ligand delivery to lysosomes, (A) and the endosome maturation model of ligand delivery to lysosomes, (B). In the preexisting compartment model (A), ligands accumulate in sorting endosomes until the rates of ligand flux in and out of the sorting endosome are equal. In the maturation model (B), ligands accumulate in sorting endosomes until the sorting endosome loses the ability to fuse with endocytic vesicles.

edly bud off from the sorting endosome while lysosomally targeted ligands are accumulated in the sorting endosome (Dunn et al., 1989). Lysosomally targeted ligands are next found in late endosomes, which are functionally distinct from sorting endosomes in that they contain very low amounts of most recycling receptors (Geuze et al., 1983; Schmid et al., 1988). While late endosomes appear incapable of fusing with newly formed endocytic vesicles (Salzman and Maxfield, 1989), they accept traffic from the Golgi apparatus, including vesicles containing lysosomal enzymes and the mannose-6-phosphate receptor (Griffiths et al., 1988; Geuze et al., 1988). Ultimately, ligands are found in hydrolytic lysosomes, where they are degraded.

At present there is no standard nomenclature for the different organelles involved in endocytosis. The terminology used in this paper is consistent with previous work from this laboratory (e.g., Salzman and Maxfield, 1989). Compartments which have been termed "early endosomes" on the basis of labeling during brief exposures to probes (e.g., Schmid et al., 1988; Gorvel et al., 1991) are equivalent to primary endocytic vesicles and sorting endosomes discussed in this paper. "Early endosomes" defined as containing transferrin (e.g., Schmid et al., 1988; Chavrier et al., 1991) will consist of primary endocytic vesicles, sorting endosomes, and recycling endosomes as defined here. The "compartment of uncoupling of receptor and ligand" or "CURL", first described by Geuze et al. (1983) is equivalent to the sorting endosome as defined here.

In the biosynthetic pathway, protein processing occurs as substrates are sequentially shuttled via vesicular transport through a series of stable, functionally distinct compartments consisting of the ER and the Golgi stacks (Palade, 1975). However, it is not yet clear if the endocytic pathway consists entirely of stable, persistent structures, analogous to Golgi cisternae or if certain endosomes might mature from

one type to another. It is clear that recycling receptors are removed from sorting endosomes by repeated budding (Dunn et al., 1989). However, it is not clear if lysosomally targeted ligands are similarly repeatedly removed from sorting endosomes and delivered to late endosomes, or if sorting endosomes themselves mature into late endosomes. Helenius et al. (1983) first posed these two alternative models for the delivery of ligands from endosomes to lysosomes, which they termed the "vesicle shuttle model" and the "maturation model", respectively. Review articles making arguments in favor of each model have recently been published (Griffiths and Gruenberg, 1991; Murphy, 1991). In the work presented here we have sought to distinguish between these two models of the endocytic apparatus by analyzing the sequential appearance of LDL in sorting endosomes and late endosomes.

Schematic diagrams of two models of the endocytic process are shown in Fig. 1. In the "pre-existing compartment model" (Fig. 1 A), ligands are first internalized into endocytic vesicles which fuse with a preexisting, stable sorting endosome. In the sorting endosome receptors are removed in recycling endosomes which repeatedly bud off to return receptors to the cell surface. Lysosomally targeted ligands are similarly removed in shuttle vesicles which repeatedly bud off and deliver their contents to late endosomes. According to this model the sorting endosome is a long-lived compartment.

In the "endosome maturation model" (Fig. 1B), ligands are delivered to sorting endosomes where receptors are removed in recycling endosomes that repeatedly bud off to return receptors to the cell surface, but lysosomally targeted ligands are retained and accumulated in the sorting endosome. With time the sorting endosome loses its capacity to fuse with endocytic vesicles, thus losing its ability to serve as a sorting endosome. The fusion-incompetent sorting endosome then matures into a late endosome. In this model,

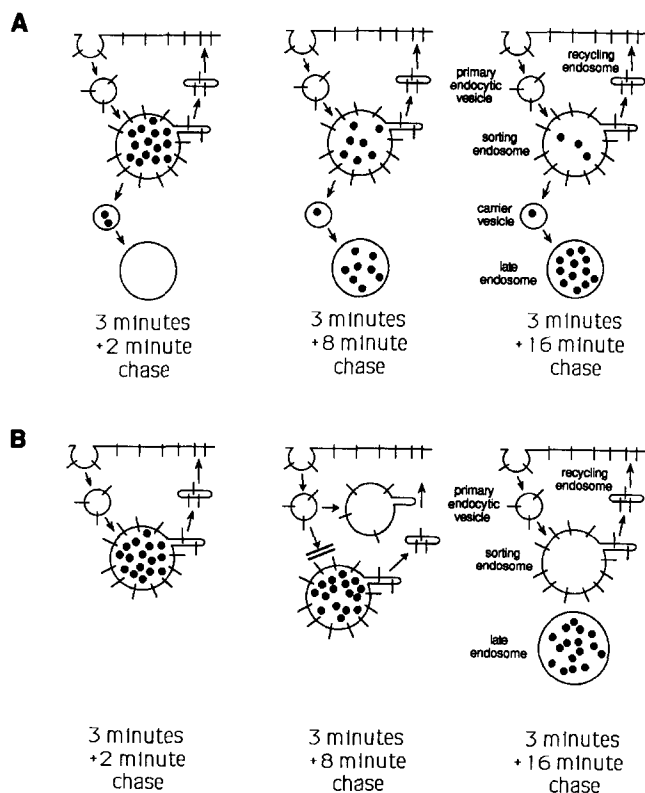


Figure 2. A pulse-chase experiment is depicted here in which cells are incubated with ligand for 3 min, and then chased for various intervals of time. The chase times of 8 and 16 min shown here represent one and two half-times of internalized ligand accessibility to subsequently endocytosed probes (Salzman and Maxfield, 1989). In the preexisting compartment model (*A*), internalized ligands become inaccessible to newly endocytosed probes with a half time of 8 min because ligands have an average residence time of 8 min in the sorting endosome before being shuttled to fusion inaccessible transport vesicles or late endosomes. In this example, ligand concentration in the sorting endosome is shown to decrease by half after 8 min and by half again after 16 min. According to the endosome maturation model (*B*), ligands become inaccessible to newly endocytosed probes as the compartments containing them lose the ability to fuse with endocytic vesicles with a half-time of 8 min. In contrast to the preexisting compartment model, the endosome maturation model predicts that the amount of ligand in each sorting endosome will not decrease. New sorting endosomes continuously form to replace those which have matured into late endosomes.

lysosomally targeted ligands are not transported in shuttle vesicles to late endosomes, but rather the endosome transforms from a sorting endosome into a late endosome. An important consequence of endosome maturation is that new sorting endosomes continuously form to replace sorting endosomes that have matured into late endosomes.

These two models make several quantitatively different predictions for the distribution of ligands in pulse-chase labeled cells which allow the two models to be experimentally distinguished. Fig. 2 depicts a pulse-chase experiment in which cells are incubated with a fluorescent ligand for 3 min and then incubated in the absence of ligand for various periods of time. Salzman and Maxfield (1989) showed that lysosomally directed α_2 -macroglobulin internalized by CHO cells becomes inaccessible to subsequently endocytosed probes with a half-time of ~ 8 min. In Fig. 2 *A* we illustrate

the properties of the preexisting compartment model with rates of ligand efflux from the sorting endosome such that the half-time of ligand residence in a sorting endosome is 8 min. Thus, after 8 min of chase in the absence of label, each sorting endosome will contain half of its original contents of ligand. In the illustration of the endosome maturation model shown in Fig. 2 *B*, sorting endosomes lose their ability to receive newly endocytosed material with a half-time of 8 min. Thus, after 8 min of chase, only half of the originally labeled sorting endosomes will be capable of fusion. In contrast to the preexisting compartment model scenario, however, the average amount of ligand in any sorting endosomes that remain fusion competent at the end of the chase period will not be reduced. Either of these models could explain the loss of fusion accessibility as ligands move to late endosomes.

Our experimental protocol was similar to that illustrated in Fig. 2, except that at the end of the chase period, cells were briefly incubated with a ligand labeled with a different fluorophore. Sorting endosomes which are fusion competent at the end of the chase interval can thus be identified by the presence of this second fluorophore. Using quantitative fluorescence microscopy of cells labeled according to this protocol we have assessed four parameters for which the two models make different experimental predictions (Table I).

Materials and Methods

Cell Cultures

TRVb-1 cells, a CHO cell line lacking endogenous Tf receptor activity and expressing transfected human Tf receptor ($\sim 150,000$ per cell) (McGraw et al., 1987), were chosen for these studies for comparability with previous studies from this laboratory (e.g., Salzman and Maxfield, 1988, 1989; Dunn et al., 1989). Cells were cultured on plastic tissue culture dishes in bicarbonate-buffered F-12 supplemented with 5% FCS (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ humidified air atmosphere. Cells were passaged by trypsinization.

2 d before each experiment cells were plated onto cover slip bottom dishes, 35-mm plastic tissue culture dishes whose bottoms have been replaced with polylysine-coated glass coverslips (Salzman and Maxfield, 1988). 1 d before each experiment, the culture medium of each was replaced with bicarbonate-buffered F-12 made with 5% delipidated FCS to stimulate increased expression of the cells' LDL receptors (Goldstein et al., 1983).

Fluorescent Ligand Preparation

LDL was prepared from whole human serum as described by Goldstein et al. (1983). DiI-LDL and diO-LDL (LDL conjugated to 3,3'-dioctadecylindocarbocyanine and 3,3'-dioctadecyloxycarbocyanine, respectively; Molecular Probes, Eugene, OR) were prepared as described by Pitas et al. (1981). Human Tf (Sigma Chemical Co., St. Louis, MO) was iron loaded, further purified by Sephacryl S-300 gel filtration, and conjugated to rhodamine as previously described (Yamashiro et al., 1984).

Fluorescent Labeling of Cells

5 min before labeling, the culture medium of each coverslip bottom dish was replaced with F-12 without bicarbonate, buffered with 20 mM Hepes (pH 7.4), supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM glucose, and 2 mg/ml ovalbumin (Sigma Chemical Co.), and incubated in air on a warm tray at 37°C. Cells were rinsed in the same medium and then incubated with 20 μ g/ml diO-LDL in 10 mM Hepes-buffered F-12 at 37°C in air for 3 min. The cells were then rinsed, incubated in the absence of label for 2, 6, 10, or 18 min, after which they were incubated with 2 μ g/ml diI-LDL for 2 min, then rinsed, and incubated in the absence of label for 2 min. (Cells were incubated with 10 times more diO-LDL than diI-LDL in order to generate similar endosome fluorescence intensities despite the greater fluorescence intensity of diI-LDL relative to diO-LDL.) Finally the

Table I. Experimental Predictions of the Preexisting Compartment and Endosome Maturation Models of Ligand Transport to Late Endosomes

Properties	Pre-existing compartment model	Endosome maturation model
Ligand content per sorting endosome	For each 8 min of chase, mean contents will decrease by half as ligands are transported to late endosomes	No change during chase
Fraction of endosomes labeled with first probe that are accessible to second probe	No change during chase	For each 8 min of chase, fraction will decrease by half as individual sorting endosomes lose fusion competence
Number of endosomes labeled with second probe that contain no first probe	No change during chase	Increasing number with time of chase as new sorting endosomes form
Number of endosomes labeled with first probe	Increases during chase period as probe becomes divided between sorting and late endosomes	No change during chase

cells were rinsed in isotonic medium 1 (150 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂), and fixed for 2 min at room temperature in 2% formaldehyde freshly diluted in medium 1, rinsed four times in medium 1 and placed in darkness until used. These experiments were repeated on three occasions with similar results.

To disturb cell physiology as little as possible, we have chosen not to strip fluorescent LDL from the surface of cells after labeling. In this protocol, if residual diO-LDL remains on the cell surface at the end of the chase interval, both diO-LDL and diI-LDL might be collected in the same coated pit and internalized into the same endocytic vesicle. Although the rate of internalization of LDL by CHO cells has not, to our knowledge, been rigorously measured, Anderson et al. (1977) report that after 2 min of internalization, 25% of the LDL-ferritin originally bound to the surface of human fibroblasts remains on the cell surface. Thus, in our 2-min chase condition co-internalization could artificially boost our measurements of endosome fusion competence. However, endosomes that contain both labels due to co-internalization would show dim diO fluorescence, as they would be labeled with only residual amounts of surface-bound diO-LDL. Thus, if the large amount of probe colocalization we find at short chase times resulted from probe co-internalization, the diO-LDL fluorescence of endosomes containing both probes should be relatively dim. However, as shown in Fig. 4, the mean diO fluorescence of sorting endosomes of cells chased for 2 min is not significantly lower than that at any subsequent period, and the distribution of sorting endosome fluorescences for cells chased for 2 min is indistinguishable from that for cells chased for 10 min. Thus, our data indicate that co-internalization (if it occurred) did not affect our measured quantities.

For studies designed to identify newly formed sorting endosomes, cells were incubated in 2 μ g/ml diI-LDL for 3 min, rinsed, and incubated in the absence of label for 10 min, incubated in 20 μ g/ml diO-LDL for 5 min, and incubated without label for a further 2 min. Cells were fixed before analysis. Cells were imaged as described previously, except that diI-LDL was excited with and without a 5% neutral density filter. By this method diI-LDL could first be imaged in the linear range of the camera, and then imaged with 20 times greater sensitivity.

In separate studies, competition of the fluorescent LDLs used in these studies with 100 \times unlabeled LDL reduced fluorescent LDL uptake to undetectable levels.

Fluorescence Microscopy

DiI-LDL was visualized using a Leitz Diavert fluorescence microscope equipped with a 63 \times , N.A. 1.4 objective, a 530–560-nm band pass excitation filter, a 580-nm dichroic mirror and 580-nm-long pass emission filter. DiO-LDL was visualized as above, but with a 450–490-nm band pass excitation filter, a 510-nm dichroic mirror, and a 515–545-nm band pass emission filter. Images were recorded on a JVC CR6650U video cassette recorder with a Videoscope VS2000N camera and a Videoscope KS1380 image intensifier. Neutral density excitation filters (50, 25, 10, 5, 2.5% transmission) were used to keep diO-LDL fluorescence intensities from exceeding the camera's linear range. The image intensifier was kept at one gain setting for all time point recordings for each ligand. Since diI-LDL fluorescence intensities were not to be quantified, higher image intensifier gain levels were used to increase sensitivity. For each field, a series of 15–20 serial

focal plane images was recorded, first for diO-LDL then, after switching filter sets, for diI-LDL. Serial focal planes were 0.8 μ m apart and were illuminated for \sim 2 s apiece. Focal plane adjustment was made using a microstepping motor z-axis controller (Kinetek, Yonkers, NY).

Image Processing

Images were digitized and background corrected as described previously (Dunn et al., 1989; Maxfield and Dunn, 1989). Using a previously described image processing technique which identifies the focused fluorescence image for each endosome in a field, distinguishes the image of each endosome from those of nearby endosomes and quantifies the fluorescence intensity of each resolved endosome (Dunn et al., 1989), a single composite image of focused endosomes was created from the set of serial focal sections collected for each probe. The fluorescence intensity of each endosome in the diO-LDL fluorescence image was quantified as the sum of the intensity values of all pixels whose brightness was at least 50% of that of the brightest pixel in the endosome image, a parameter that was found to be relatively insensitive to focus errors. Measurements of serial focal plane images (taken at 0.1- μ m intervals) of 0.13- μ m fluorescent microspheres showed that measured intensity errors would be restricted to \sim 5% by this technique. Low illumination intensities were employed to minimize photobleaching during the multiple exposures involved in serial focal plane imaging. In separate studies we determined that the diO-LDL fluorescence of endosomes located in the final focal planes imaged for each field would be attenuated by \sim 10% due to photobleaching. Fluorescence images of diI-LDL-labeled endosomes were likewise trimmed to include only those pixels whose intensities were at least 50% of that of the brightest pixel in each endosome image. This procedure removes all but the central 25% of the original endosome image, on average. Endosomes containing both diO-LDL and diI-LDL were identified as those whose diO and diI image centers spatially overlapped when the two images were combined. To estimate the amount of measured colocalization that results from random coincidence of closely adjacent, but distinct diO-LDL- and diI-LDL-containing endosomes, randomization tests were performed in which diO and diI images were combined after randomly offsetting one relative to the other (over a range of 0.5–2.3 μ m). These tests indicated that random coincidence will account for an average of 12% colocalization found for diO-LDL-containing endosomes.

Results

Ligand Content per Sorting Endosome: diO-LDL Is Retained by Individual Sorting Endosomes during a Chase

TRVb-1 cells were incubated in diO-LDL for 3 min, incubated in the absence of label for various periods of time, and then incubated in diI-LDL for 2 min. The cells were then further incubated in the absence of label for 2 min to chase

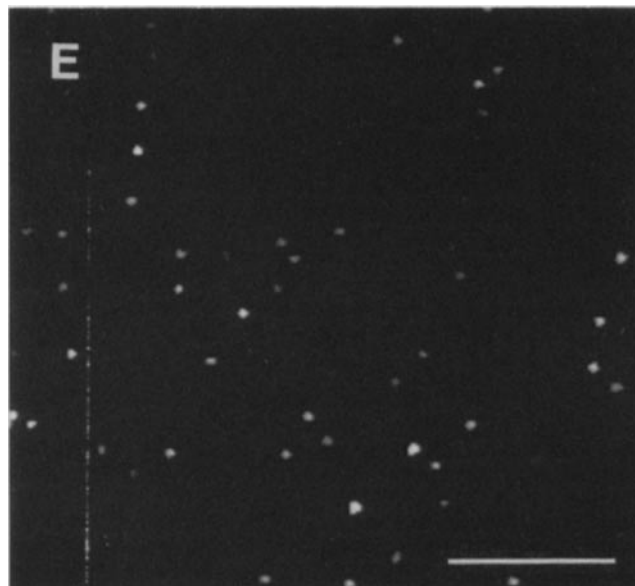
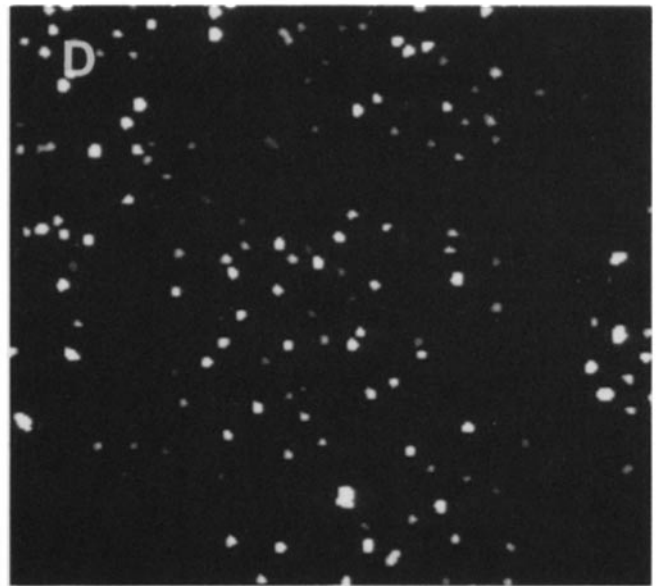
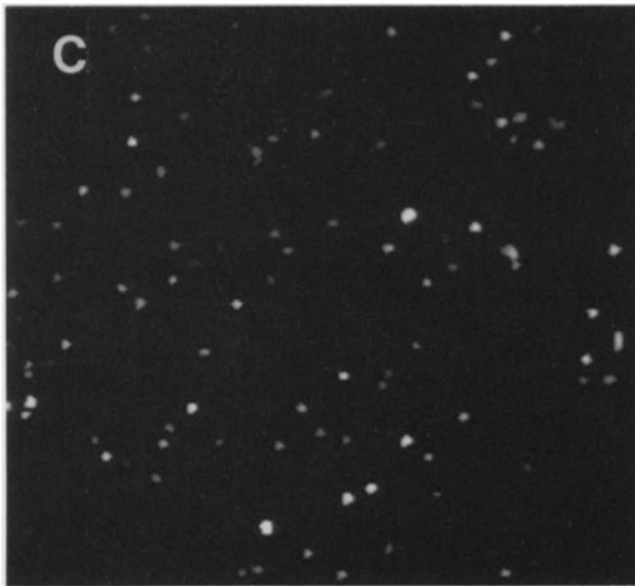
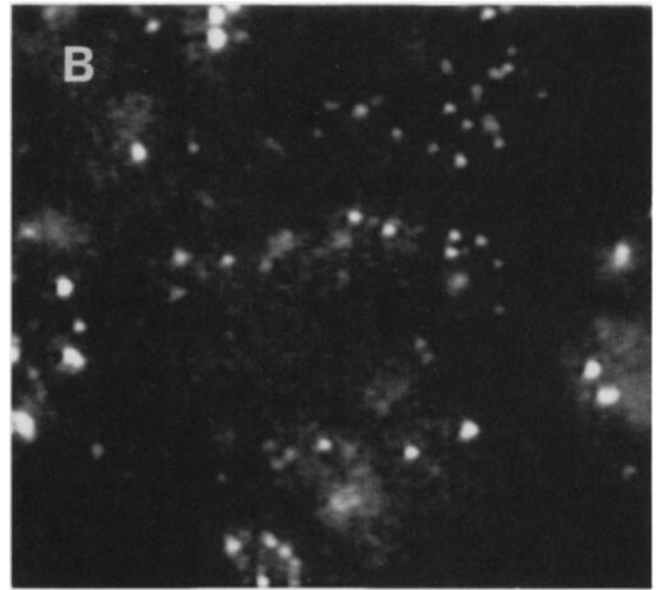
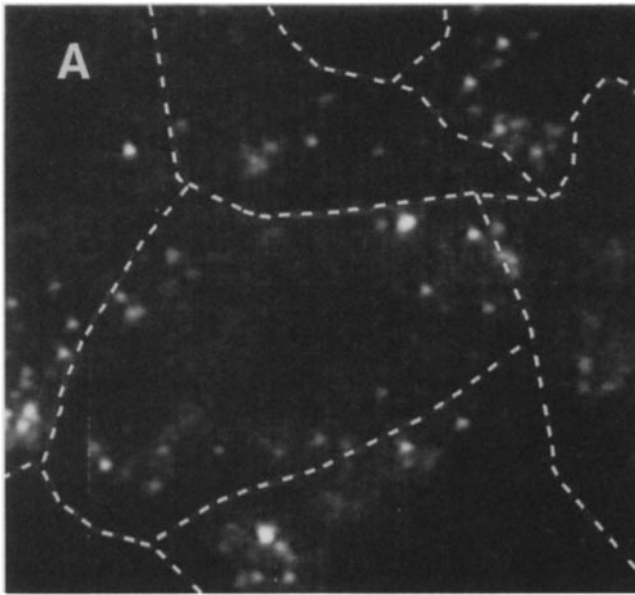


Figure 3. TRVB-1 cells were incubated in diO-LDL for 3 min, then incubated in the absence of label for 2 min, then incubated in diI-LDL for 2 min, and finally incubated in the absence of label for 2 min. (A) DiO-LDL and (B) DiI-LDL fluorescence images, each from a single focal plane. (C) Processed composite diO-LDL fluorescence image and (D) processed composite diI-LDL fluorescence image, each resulting from combining the background-corrected, focus-corrected images from all focal planes. (E) Processed composite diO-LDL fluorescence image of endosomes containing both diO-LDL and diI-LDL. Cell borders are indicated by dashed lines in A. Bar, 10 μm .

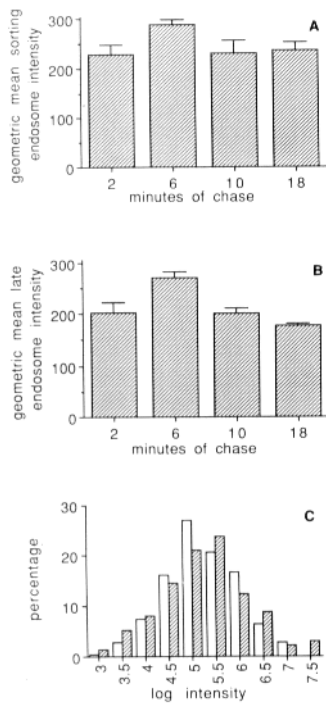


Figure 4. Geometric mean diO-LDL fluorescence intensities (arbitrary units) of (A) sorting endosomes and (B) late endosomes after chase periods of varying durations. Cells were first labeled with diO-LDL for 3 min, chased for the indicated intervals of time, labeled with diI-LDL for 2 min, and finally chased for another 2 min. Indicated values are geometric means (\pm SE) of the diO-LDL fluorescence of six fields, each containing an average of seven cells each. This experiment was repeated on three separate occasions with similar results in each case. (C) Log-normal frequency distributions of sorting endosome diO-LDL fluorescence intensities from images of cells chased for either 2 min (open bars, $n = 329$) or 10 min (hatched bars, $n = 138$).

diI-LDL into sorting endosomes. The various stages of image processing conducted on the fluorescence images of cells labeled with diO-LDL and chased for 2 min before incubation in diI-LDL are depicted in Fig. 3. Brief labeling of TRVb-1 cells with fluorescent LDL results in a punctate pattern of fluorescence (Dunn et al., 1989) in either fixed or living cells, with no evidence of a reticular endosomal network of the kind described by Hopkins et al. (1990) in living HEP2 cells. Our instrumentation can easily detect such tubular endosomes, since we find that brief incubations of HEP2 cells in fluorescent transferrin results in labeling of a reticular endosomal network (not shown here). In CHO cells, transferrin initially colocalizes with LDL, but it is rapidly removed to a collection of tubular recycling endosomes near the Golgi apparatus (Dunn et al., 1989; Yamashiro et al., 1984).

The preexisting compartment model predicts that sorting endosomes would repeatedly export endocytosed LDL to late endosomes. In our pulse-chase-pulse experiments, this characteristic would be reflected in a decrease in diO-LDL fluorescence in sorting endosomes, with a concomitant increase in the amount of label in late endosomes (see Fig. 2). As shown in Fig. 4 A, the geometric mean diO-LDL fluorescence of sorting endosomes (endosomes which are accessible to endocytosed diI-LDL immediately after internalization) remains nearly constant over a 16-min period. In Fig. 4 B, the geometric mean of diO-LDL fluorescence of late endosomes (endosomes that are not accessible to newly endocytosed diI-LDL) shows no sign of an increase over this interval. Fig. 4 C shows a comparison of the diO-LDL fluorescence intensity distribution of sorting endosomes for cells chased for either 2 or 10 min. Both the means and the distributions of fluorescence intensity remain essentially unchanged over this time. Taken together, these results indicate that LDL is not slowly exported from sorting to late endosomes.

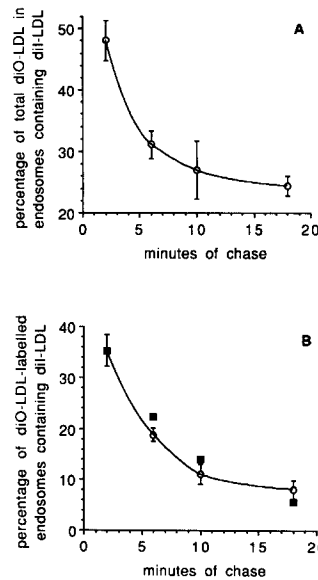


Figure 5. Fusion accessibility of diO-LDL to subsequently endocytosed diI-LDL as a function of the duration of the chase interval between administration of the two probes. Data were derived from the same cells described in Fig. 4. (A) The fraction of total internalized diO-LDL found in compartments labeled with diI-LDL. (B) The fraction of diO-LDL-containing compartments which are labeled with diI-LDL. The values in B have been corrected for random coincidence of diO-LDL and diI-LDL (see Materials and Methods). For comparison, squares in B depict exponential decay in sorting endosome fusion accessibility with a half-time of 6 min.

Fraction of Endosomes Labeled with First Probe That Are Accessible to Second Probe: Endosomes Labeled with diO-LDL Lose the Capacity to Receive Subsequently Internalized diI-LDL

Using the same fields of cells described above, we can quantify the fusion accessibility of LDL as a function of time after internalization by quantifying the fraction of diO-LDL that is present in endosomes that also contain subsequently endocytosed diI-LDL. It should be noted that this fraction will be less than 100% at the start of the measurement as a result of ongoing cytoplasmic processing during the preliminary incubations. In addition, some of the diI-containing endosomes may be lost during image processing. As shown in Fig. 5 A, the fusion accessibility of endocytosed diO-LDL to subsequently endocytosed diI-LDL rapidly decreases with time. Although this result is consistent with either the shuttle vesicle model or the maturation model, when these data are presented as the fraction of diO-LDL-containing endosomes which also contain diI-LDL (Figs. 5 B and 6), essentially identical kinetics are obtained. These results suggest that endocytosed diO-LDL becomes inaccessible to subsequently endocytosed diI-LDL as the diO-LDL-containing endosomes themselves lose the capacity to fuse with newly formed endocytic vesicles with time.

Number of Endosomes Labeled with Second Probe That Contain No First Probe: New Sorting Endosomes Are Formed during a Chase

The maturation model predicts that the population of sorting endosomes is continuously being depleted by the maturation of sorting endosomes into late endosomes. A corollary prediction is that new sorting endosomes must continuously form to compensate for this loss. In cells labeled according to the pulse-chase-pulse-chase protocol used in these studies, the new sorting endosomes predicted by the endosome maturation model may be identified as those containing only the second probe since these endosomes will have formed after the removal of the first probe from the incuba-

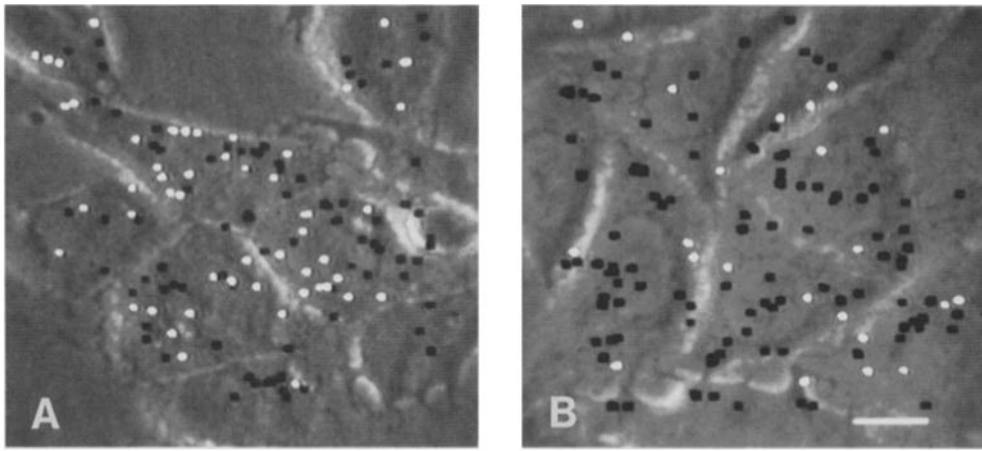


Figure 6. Overlaid fluorescence and phase-contrast images of cells labeled for 3 min with diO-LDL, then chased for (A) 2 min or (B) 10 min before a 2-min pulse of diI-LDL and a 2-min chase. Endosomes containing only diO-LDL are depicted in black. Endosomes containing both diO-LDL and diI-LDL are depicted in white. Bar, 10 μ m.

tion medium. According to the preexisting compartment model, endosomes containing the second probe will also contain first probe, but the amount of first probe remaining may be small. Thus, experimentally distinguishing between these two models requires that we be able to detect what may be very small residual amounts of the first probe in endosomes containing the second probe. The studies described thus far were designed to maximize our ability to detect sorting endosomes, and thus were designed for maximum sensitivity to the second probe. To sensitively test for the presence of first probe in endosomes containing second probe the previously used experimental protocol was altered (see Materials and Methods).

In the revised protocol, diO-LDL was used as the second probe and the brighter diI-LDL was used as the first probe. By collecting two sets of images of diI-LDL fluorescence, one adjusted to fit the linear range of the video camera, and the other at 20 times that intensity, our sensitivity for the first probe was increased so that we were capable of detecting dim endosomes which contained as little as 1% of the mean endosome contents of diI-LDL. A representative field of cells labeled by this procedure is shown in Fig. 7. Several fields were analyzed, and all showed that a sizeable fraction of the endosomes containing diO-LDL contain no detectable diI-LDL (<1% of the original mean endosomal contents). Representative endosomes containing diO-LDL but not diI-LDL are indicated by arrows in Fig. 7 (B and D). We were not able to assign a value to the fraction of diO-LDL-containing endosomes containing undetectable amounts of diI-LDL since increasing the fluorescence signal by 20-fold causes significant camera "blooming" of high intensity signals (spilling of high intensity signals into adjacent regions of the image), which will have the effect of obscuring dim diI-LDL-containing endosomes which are near very bright ones.

Number of Endosomes Labeled with First Probe: Constant Number during 16-min Chase

According to the endosome maturation model, after accumulation in sorting endosomes, endocytosed LDL will be retained in these compartments as they mature into late endosomes. Consequently, the number of endosomes containing a pulse of LDL should remain constant during a chase. In contrast, the preexisting compartment model predicts that the number of labeled compartments will fluctuate as a pulse

of LDL is first apportioned to sorting endosomes, is next transferred increasingly to late endosomes as well, and is finally found only in late endosomes. This reapportionment would be reflected in a fluctuation in the ratio of the number of endosomes containing the first pulse of LDL (diO-LDL) to the number of sorting endosomes containing the second pulse of LDL (diI-LDL). In fact, we find that the ratio of the number of endosomes labeled with diO-LDL to the number of endosomes labeled with diI-LDL is essentially constant during a 16-min chase (Fig. 8).

Discussion

Because of the relative ease with which endocytic compartments can be labeled with fluorescent or electron dense markers, the itineraries of endocytic ligands are well documented (see Goldstein et al., 1985 for review). However, the mechanisms underlying these itineraries are not well understood. The experiments presented here have been designed to distinguish between two models of ligand trafficking from sorting endosomes to late endosomes, which have been termed the "preexisting compartment" and the "endosome maturation" models. In the preexisting compartment model, ligands are transferred from sorting endosomes to late endosomes via carrier vesicles during the process of sorting. In the endosome maturation model, ligands are transferred to late endosomes as sorting endosomes are transformed into late endosomes.

Both models are consistent with results from previous studies of the sorting endosome. We have previously found that in CHO cells, LDL accumulates in the sorting endosome for a period of \sim 10 min, but that transferrin accumulates for only 2–3 min and to a much smaller extent (Dunn et al., 1989). According to the preexisting compartment model, this accumulation could result from a balance between ligand influx and efflux rates such that the concentration of lysosomally directed ligand in the sorting endosome increases for \sim 10 min (see Fig. 1A). Since the rates of budding of recycling endosomes and carrier vesicles need not be the same, the preexisting compartment model could lead to different accumulation kinetics for LDL vs. transferrin. In the endosome maturation model, lysosomally directed ligands are retained by sorting endosomes and thus accumulate during the period in which sorting endosomes are capa-

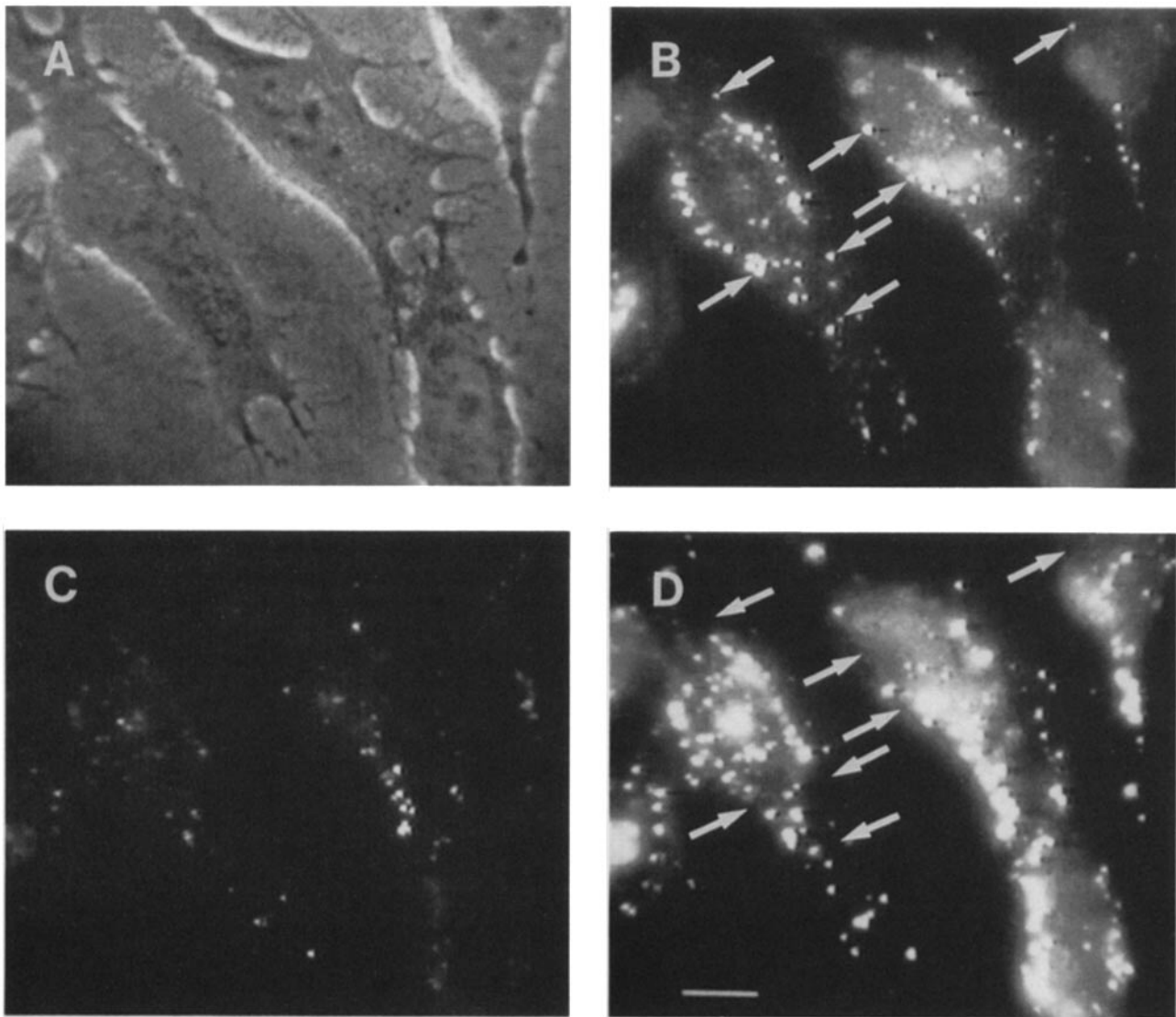


Figure 7. Formation of new sorting endosomes. A representative field of cells is shown from an experiment in which cells were labeled for 3 min with diI-LDL, then chased for 10 min before a 5-min pulse of diO-LDL and a 2-min chase. (A) Phase-contrast image. (B) DiO-LDL fluorescence. (C) DiI-LDL fluorescence imaged in the linear detection range of the video camera. (D) DiI-LDL fluorescence imaged at 20 \times greater sensitivity. Arrows placed in regions unaffected by camera blooming indicate endosomes that contain diO-LDL but not diI-LDL. Bar, 10 μ m.

ble of fusing with endocytic vesicles (see Fig. 1 B). Since transferrin is repeatedly removed from the sorting endosome, it accumulates only briefly and slightly.

Salzman and Maxfield (1989) have shown that when CHO cells are pulse labeled with ligands destined for delivery to lysosomes, these ligands become inaccessible to subsequently endocytosed probes with a half-time of \sim 8 min. Numerous other studies of endosome fusion both in vivo (Salzman and Maxfield, 1988; Ward et al., 1990; Stoorvogel et al., 1991) and in vitro (Gruenberg and Howell, 1989) have also shown that while endocytosed probes are initially accessible to subsequently internalized probes, fusion accessibility rapidly decreases with time. Endocytosed ligands may become inaccessible to subsequently endocytosed probes either as they are shuttled to late endosomes, as in the pre-existing compartment model, or as the sorting endosome containing the ligands loses its capacity for fusion, as in the maturation model.

The studies described here take advantage of the different predictions made by each model for several measurable parameters in pulse-chase-pulse studies. These parameters are uniquely measurable by quantitative fluorescence microscopy which allows characterization of individual endosomes. By quantitatively analyzing the sequential appearance of LDL in individual sorting endosomes and late endosomes in pulse-chase-pulse studies, we have obtained results which, by the criteria listed in Table I, are inconsistent with a preexisting compartment model and support a maturation model.

The average amount of fluorescent LDL in individual sorting endosomes does not decrease during the course of a chase, nor does the average amount of LDL in individual late endosomes increase. These data indicate that once loaded into sorting endosomes, lysosomally targeted ligands, such as LDL, are retained there. This is in contrast to transferrin, which is repeatedly removed from sorting endosomes (Dunn

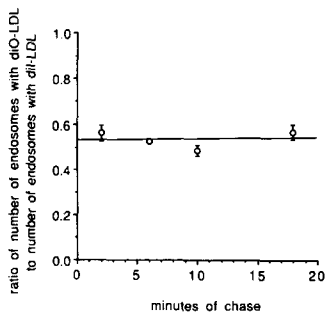


Figure 8. Constancy of number of compartments labeled with a pulse of diO-LDL during the ensuing chase. Data were derived from the same images described in Fig. 4. Data are expressed as the ratio of the number of endosomes containing diO-LDL to the number of endosomes labeled with diI-LDL. The higher camera gain used to image diI-

LDL resulted in a greater number of endosomes detected containing diI-LDL as compared with diO-LDL (see Materials and Methods).

et al., 1989). A corollary observation is that we find no increase in the number of compartments labelled with LDL; the number of compartments labeled during a pulse is stable during an ensuing chase. The constant amount of diO-LDL found in individual sorting endosomes is consistent with all of the diO-LDL within an endosome becoming fusion inaccessible simultaneously, as in the maturation model, not by repeated budding off of vesicles, as in the preexisting compartment model. Furthermore, the levels of diO-LDL in late endosomes are, at all times, similar to those in sorting endosomes, showing no signs of an accumulation, as would be expected in the preexisting compartment model. These data are most easily explained by the maturation of each sorting endosome into a late endosome.

The fusion accessibility of endocytosed diO-LDL to subsequently endocytosed diI-LDL decreases rapidly with time. Despite the different approach used here, this result is similar to that of Salzman and Maxfield (1989) who found that the fusion accessibility of endocytosed α_2 -macroglobulin decreases with time. The decrease in fusion accessibility of diO-LDL does not result from the repeated transfer of diO-LDL from fusion-competent sorting endosomes to fusion-incompetent late endosomes since, as reported above, diO-LDL is retained by the sorting endosome. By characterizing the fusion accessibility of individual endosomes, we have demonstrated that the diO-LDL-containing endosomes themselves lose the ability to fuse with newly formed endocytic vesicles with time. Thus, these results extend those of Salzman and Maxfield (1989) and indicate that endocytosed ligand accessibility to subsequently endocytosed probes decreases with time because sorting endosomes become incapable of fusing with newly formed endocytic vesicles.

With time, newly endocytosed diO-LDL is increasingly directed to endosomes lacking detectable levels of previously endocytosed diI-LDL indicating that new sorting endosomes must continuously form. These data suggest that the sorting endosome is a transient organelle, whose constant numbers in a cell result from a balance between the formation of new sorting endosomes and the depletion that results from maturation of sorting endosomes into late endosomes.

The question of how ligands are transported from sorting endosomes to late endosomes has recently been studied by Stoorvogel et al. (1991), who provide evidence that sorting endosomes transform into late endosomes. Examination of ultrathin cryosections indicates a gradual inverse relationship between the number of transferrin-HRP molecules and

the number of mannose-6-phosphate receptors in individual endosomes. The continuous gradation of each label suggests that individual sorting endosomes lose transferrin and acquire mannose-6-phosphate receptor, a marker for late endosomes, thus maturing into late endosomes. However, it should be pointed out that some of the transferrin-HRP detected in later compartments may represent transferrin misdirected to lysosomes, since previous work from this laboratory (Stoorvogel et al., 1988) has shown that conjugation to horseradish peroxidase slightly decreases the recycling efficiency of transferrin. Stoorvogel et al. (1991) also found that an ensemble of endosomes labeled with asialoorosomucoid gradually increased in density, approaching the density of lysosomes. Although these data are consistent with a maturational increase in the density of individual sorting endosomes, they could also result from a slow transfer of asialoorosomucoid from stable sorting endosomes to late endosomes, resulting in a shift in mean endosome density with no change in any individual endosome density. However, Roederer et al. (1990) have found that isolated endosomes are capable of autonomously increasing in density to a density characteristic of lysosomes.

Our studies do not directly address the question of whether individual sorting endosomes mature gradually or suddenly, but our data suggest that the loss of fusion competence by a sorting endosome occurs suddenly. If sorting endosomes gradually lose the capacity to fuse with endocytic vesicles, it would be expected that longer chase periods would result in diminishing amounts of second probe being delivered to endosomes containing first probe. Although our studies were not designed to measure this quantity, qualitative inspection indicates that the amount of second probe in doubly labeled endosomes appears to be independent of time. Furthermore, in studies of early and late endosomes isolated via free-flow electrophoresis, the absence of intermediate forms suggests that the transition occurs abruptly rather than gradually (Schmid et al., 1988).

Although our data do not support the preexisting compartment model in which lysosomally directed ligands are repeatedly withdrawn from the sorting endosome, they are not capable of distinguishing between a maturation model and an extreme form of the preexisting compartment model in which all, or nearly all of the sorting endosome's lysosomally directed contents are withdrawn in single, infrequent events. Gruenberg et al. (1989) suggest that large (0.5 μ m) spherical vesicles may act as carrier vesicles, shuttling ligands from sorting endosomes to late endosomes. However, if such withdrawals were to occur, they would have to be highly efficient; in pulse-chase-pulse studies we find that after a 10-min chase, a large fraction of sorting endosomes contains levels of the first probe below our detection limits (<1% of the original average endosome contents). Although the process of formation of a large shuttle vesicle may be considered to be essentially similar to the process of endosome maturation, it should be pointed out that the distinction between this form of the preexisting compartment model and the maturation model is not arbitrary. As discussed below, the two models have profoundly different implications for cellular lipid and protein routing.

The significant functional differences between sorting endosomes and late endosomes imply underlying differences in the distribution of the protein components of endosomes. Using free flow electrophoresis, Schmid et al. (1988) have

shown that the protein composition of "early endosomes" differs from that of "late endosomes". Van Der Sluijs et al. (1991) find that one of the proteins enriched in the early endosome fraction prepared by this technique is the small GTP-binding protein rab4. Chavrier et al. (1990) have localized rab7 in late endosomes and rab5 in early endosomes of BHK and MDCK cells. Gorvel et al. (1991) have demonstrated that rab5 is required for early endosome fusion in BHK cells. Although differences in the protein constitution of early and late endosomes have been interpreted as supporting a pre-existing compartment model (Griffiths and Gruenberg, 1991), it should be pointed out that a maturation process is equally capable of generating such differences (Murphy, 1991). However, the two models do have different ramifications for cellular trafficking of the functional constituents of endosomes; the endosome maturation model predicts a significant cellular traffic that would not be required in the pre-existing compartment model. If sorting endosomes mature into late endosomes, the repeated de novo formation of new sorting endosomes requires the repeated delivery of all of the functional machinery of sorting endosomes (e.g., ion channels and pumps, targeting proteins, cytoskeletal binding proteins and motor proteins). Similarly the repeated transformation of sorting endosomes into late endosomes requires repeated retrieval of the constituents of sorting endosomes, and repeated delivery of the constituents of late endosomes (e.g. mannose-6-phosphate receptor and associated ligands). Understanding the endocytic machinery may thus involve elucidating not only the pathways followed by ligands and receptors, but also what may be a substantial underlying protein traffic that serves to control functions such as acidification, vesicle fusion, vesicle budding, and endosome movement.

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