

A Prelysosomal Compartment Sequesters Membrane-impermeant Fluorescent Dyes From The Cytoplasmic Matrix of J774 Macrophages

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Abstract. After the membrane impermeant dye Lucifer Yellow is introduced into the cytoplasmic matrix of J774 cells, the dye is sequestered within cytoplasmic vacuoles and secreted into the extracellular medium. In the present work we studied the intracellular transport of Lucifer Yellow in J774 macrophages and the nature of the cytoplasmic vacuoles into which this dye is sequestered. When the lysosomal system of J774 cells was prelabeled with a Texas red ovalbumin conjugate and Lucifer Yellow was then loaded into the cytoplasm of the cells by ATP-mediated permeabilization of the plasma membrane, the vacuoles that sequestered Lucifer Yellow 30 min later were distinct from the Texas red-stained lysosomes. After an additional 30 min Lucifer Yellow and Texas red co-localized in the same membrane bound compartments, indicating that the Lucifer Yellow had been delivered to lysosomes. We next prelabeled the plasma membrane of J774 cells with anti-macrophage antibody and Texas red protein A before Lucifer Yellow was loaded into the cells. The phase-lucent vacuoles that subsequently sequestered Lucifer Yellow also stained with Texas red, showing that they were part of the endo-

cytic pathway. J774 cells were fractionated on percoll density gradients either 15 or 60 min after Lucifer Yellow was introduced into the cytoplasmic matrix of the cells. In cells fractionated after 15 min, Lucifer Yellow was contained within the fractions of light buoyant density that contain plasma membrane and endosomes; the dye later appeared in vesicles of higher density which contained lysosomes.

Secretion of Lucifer Yellow from the cytoplasmic matrix of J774 cells is inhibited by the organic anion transport blocker probenecid. We found that probenecid also reversibly inhibited sequestration of dye, indicating that sequestration of dye within cytoplasmic vacuoles was also mediated by organic anion transporters.

These studies show that the vacuoles that sequester Lucifer Yellow from the cytoplasmic matrix of J774 cells possess the attributes of endosomes. Thus, in addition to their role in sorting of membrane bound and soluble substances, macrophage endosomes may play a role in the accumulation and transport of molecules resident in the soluble cytoplasm.

THE endosomal compartment plays a central role in the intracellular transport and sorting of endocytosed materials (14), membrane proteins (17), and lysosomal enzymes (3). To date, all of the molecules that have been associated with the lumen of endosomes are those that are delivered to the endosomes by vesicular transport from the plasma membrane, the Golgi apparatus, or lysosomes. Like other cellular membranes, the membrane of the endosomal system is thought to be impermeable to water soluble molecules, and for this reason it is assumed that endosomes exclude molecules residing in the cytoplasmic matrix. Our finding of a simple and reproducible method for introducing membrane impermeant dyes into the cytoplasmic matrix of mouse peritoneal macrophages and the J774 macrophage-

like cell line provided an opportunity to examine this assumption.

Extracellular ATP permeabilizes the plasma membranes of mouse peritoneal macrophages and J774 cells to molecules ≤ 831 D (19). ATP permeabilization is mediated by ATP^{4-} and inhibited or reversed by divalent cations such as Mg^{2+} , which binds ATP^{4-} forming the chelate MgATP^{2-} (13, 18). Using this method we have filled the cytoplasmic matrix of J774 cells with membrane impermeant anionic dyes such as Lucifer Yellow, carboxyfluorescein, and fura-2. When ATP is removed from the medium, the plasma membrane regains impermeability to these dyes and they are trapped within the cytoplasmic matrix of the cells.

However, these dyes do not remain inside macrophages very long. Within 30 min, 87% of the Lucifer Yellow originally trapped within the cytoplasmic matrix is secreted into the extracellular medium, and the dye remaining within the

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cells is sequestered in phase-lucent cytoplasmic vacuoles (20). Transport of Lucifer Yellow out of the cytoplasmic matrix appears to be an active transport process, as J774 cells can clear the cytoplasm of Lucifer Yellow when the extracellular concentration of the dye is higher than the intracellular dye concentration. Furthermore, dye secretion is mediated by organic anion transporters and is inhibited by the drugs probenecid and sulfapyrazone, which block organic anion transport in renal tubular cells, hepatocytes, and other epithelia.

In the present work we investigated the intracellular pathway of Lucifer Yellow sequestration in J774 cells to determine the nature of the phase-lucent cytoplasmic vacuoles that accumulate this dye and the fate of the dye within the vacuolar apparatus. These studies identify a novel pathway that mediates the transport of organic anions from the cytoplasmic matrix into an endosome-like compartment.

Materials and Methods

Chemicals

ATP (special grade) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Lucifer Yellow CH (tetralithium salt), Texas red-labeled ovalbumin, and Texas red-labeled protein A were from Molecular Probes, Inc. (Eugene, OR). Percoll was from Pharmacia Fine Chemicals (Piscataway, NJ). Monensin, chloroquine, valinomycin, and nigericin were from Sigma Chemical Co. (St. Louis, MO). Rabbit antibody to mouse macrophages was a generous gift of Dr. Jay Unkeless (Mt. Sinai School of Medicine, New York, NY).

Cells and Media

The J774 mouse macrophage-like cell line was grown in spinner cultures in DME supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (D10F)¹ or in DME supplemented with 10% heat-inactivated calf serum. For microscopy, $1-2 \times 10^5$ cells were plated for 6-12 h on 12 mm No. 1 glass coverslips in 16-mm tissue culture wells containing D10F.

ATP Permeabilization

Cells were incubated for 5 min at 37°C in D10F containing 5 mM ATP and Lucifer Yellow as indicated, and washed in Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PD). D10F was added and cells were incubated at 37°C until required for microscopy. Cell viability was >95% after ATP permeabilization as assessed by trypan blue exclusion (1).

Incubation of J774 cells in medium containing ATP induced the appearance of large phase-lucent cytoplasmic vacuoles, as reported by Cohn and Parks (4). The chronology of the appearance of these vacuoles was as follows: J774 cells examined before or 5 min after exposure to ATP had occasional phase-lucent vacuoles. On average there were 1.4 phase-lucent vacuoles per cell 5 min after removal of ATP. A few cells had 5-10 vacuoles, and many cells contained no vacuoles. The number of vacuoles increased markedly between 5 and 10 min after ATP treatment in cells maintained at 37°C. This increase in phase-lucent vacuoles was not affected by the presence of Lucifer Yellow in the medium during ATP permeabilization. 15 min after ATP treatment, there was an average of 7.1 vacuoles per cell; many cells contained 10-20 vacuoles, but the average was lower because some cells had no discernable vacuoles. The vacuoles remained prominent until 20 min after ATP treatment, after which time they decreased in number. By 45 min the quantity of vacuoles had returned to the level existing before ATP treatment.

Fluorescence Microscopy

Cells were observed without fixation. J774 cells adherent to glass coverslips

were washed several times with PD, the coverslips were mounted on microscope slides with glass supports interposed, and the coverslip edges were sealed with liquefied paraffin-vaseline-lanolin gel (1:1:1 mixture). Cells were observed with a Zeiss Photomicroscope III, with rhodamine and fluorescein filter sets. Color photographs were taken with Ektachrome ASA 400 film, with the ASA set at 800 for fluorescein optics, and 1,600 or 3,200 for rhodamine optics. Film was push-processed to ASA 800. Photographs taken with Tri-X Pan film (ASA 400), were exposed at ASA 1,600 and processed with Diafine developer.

Subcellular Fractionation

We used a modification of the fractionation procedure developed by Gallo-way et al. for J774 cells (11). Cells were plated for 4 h on 100-mm tissue culture plates ($1-2 \times 10^7$ cells per dish), and Lucifer Yellow was introduced into the cells by incubating the cells in DME containing 10% heat-inactivated calf serum, 5 mg/ml Lucifer Yellow, and 5 mM ATP for 10 min at 37°C. The plates were washed and immersed in three beakers of PD, the first of which contained 0.1 mg/ml BSA, and fresh medium was added. The cells were incubated at 37°C for 15 or 60 min and then washed. All subsequent steps were performed at 4°C. 2.5 ml of ice cold sucrose homogenization buffer (SHB; 250 mM sucrose, 1 mM EDTA, 20 mM Hepes, pH 7.4) containing 1.0 mM PMSF and 0.6 TIU/ml aprotinin was added to each plate and the cells were harvested with rubber policemen. The cells from two plates were pooled and homogenized in a stainless steel Dounce homogenizer (Kontes) until 85-90% of the cells were disrupted as assessed by phase-contrast microscopy. Nuclei and unbroken cells were removed by centrifugation (400 g for 10 min at 4°C), and the post-nuclear supernatant was centrifuged at 18,000 g for 30 min at 4°C in a Sorvall SS-34 rotor. The resulting membrane pellet (the large particulate fraction) contained 60-70% of the beta-glucuronidase and alkaline phosphodiesterase I activities present in the post-nuclear supernatant. This pellet was resuspended in 2 ml of SHB and layered on top of 10 ml of a gradient of 27% percoll in SHB, centrifuged at 18,000 g for 60 min, and 1-ml aliquots were removed from the bottom of the gradient. Fractions were diluted with 2 ml of KCl buffer (150 mM KCl, 1 mM EDTA, 20 mM Hepes, pH 7.4) and centrifuged at 100,000 g for 60 min. The membranes from each fraction were retrieved from above the percoll pellet and resuspended in 1 ml of 0.1% Triton X-100. Lucifer Yellow content was quantitated as described above. 60-70% of Lucifer Yellow in the large particulate fraction was recovered in the final gradient fractions; leakage of dye from vesicles during centrifugation accounted for most of remaining dye. To account for autofluorescence of the fractions, parallel experiments were performed in the absence of Lucifer Yellow, and the Lucifer Yellow content of each gradient fraction was calculated as the difference between the fluorescence of the fractions from tandem experiments performed in the presence and absence of Lucifer Yellow. Alkaline phosphodiesterase I (9) and beta-glucuronidase (2) were measured as described on all fractions.

Electron Microscopy

J774 cells were plated in 16-mm tissue culture wells, incubated in medium containing 5 mM ATP at 37°C for 5 min, washed, and incubated in D10F at 37°C for 15 min. Cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer with 4.5% sucrose and 0.48 mM CaCl_2 , pH 7.4, post-fixed in 1% osmium tetroxide in cacodylate buffer, stained in block with saturated uranyl acetate in water for 20 min, and dehydrated in a graded series of ethanol solutions. Specimens were embedded in epon, sectioned on an MT 6000 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX electron microscope.

Results

Lucifer Yellow is Sequestered in Prelysosomal Vacuoles and Subsequently Delivered to Lysosomes

We have previously shown that J774 cells loaded with Lucifer Yellow by ATP permeabilization sequester the dye as illustrated in Fig. 1, *b* and *e* (19, 20). Immediately after the ATP and Lucifer Yellow were removed, the Lucifer Yellow diffusely stained the cytoplasmic and nuclear matrices (Fig. 1 *b*); phase-lucent cytoplasmic vacuoles present at this time excluded the dye (Fig. 1, *a* and *b*). 30 min after Lucifer Yel-

1. Abbreviations used in this paper: D10F, Dulbecco's modified Eagle medium supplemented with 10% FBS; PD, Dulbecco's PBS without divalent cations; SHB, sucrose homogenization buffer.

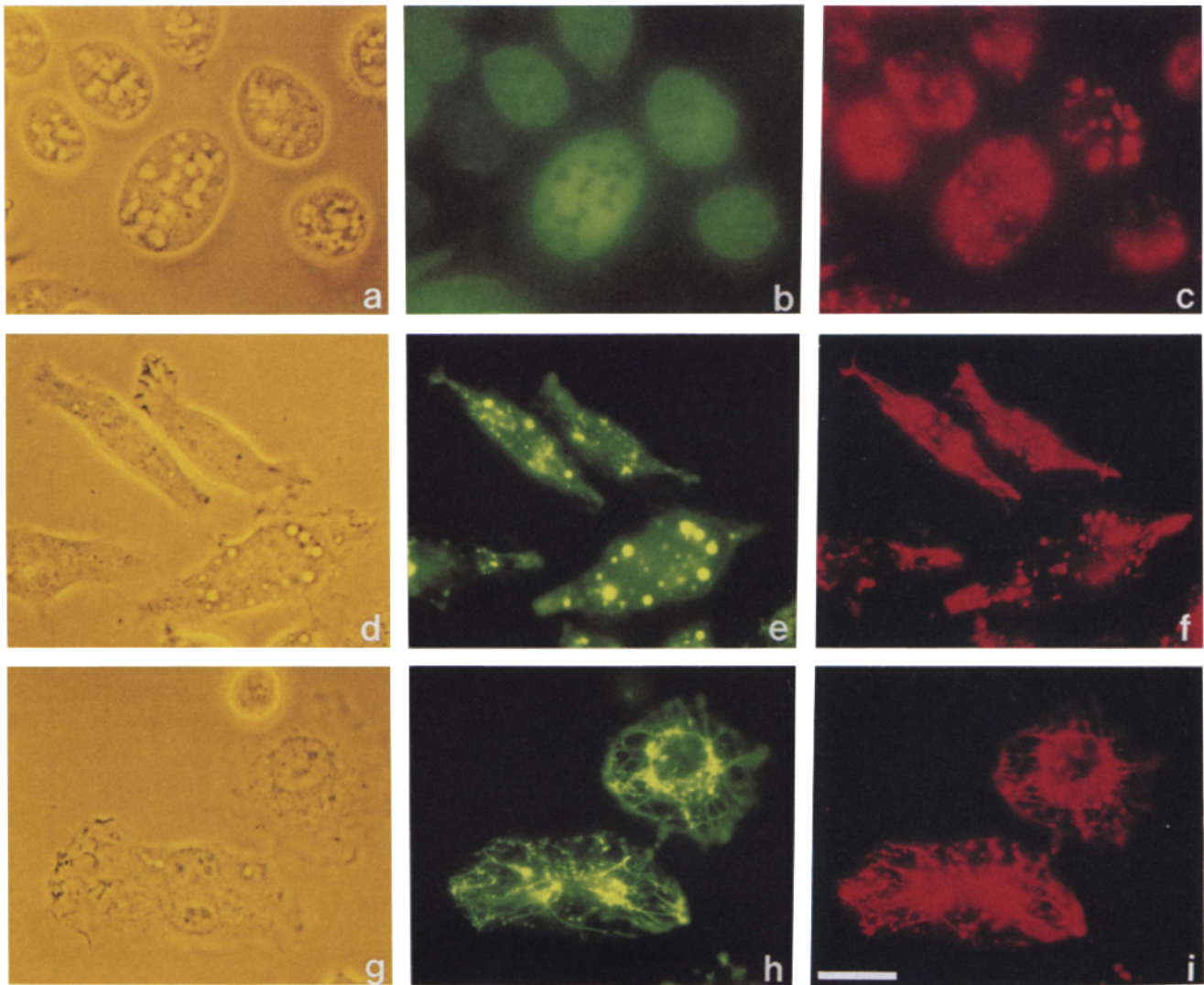


Figure 1. Lucifer Yellow introduced into the cytoplasmic matrix of J774 cells is sequestered within a prelysosomal compartment and subsequently delivered to lysosomes. J774 cells adherent to glass coverslips were incubated in D10F buffered with 10 mM Hepes (*a-c*) or bicarbonate (*d-i*) and containing 5 μ g/ml Texas red-labeled ovalbumin for 1 h, washed, exposed to D10F with 0.5 mg/ml Lucifer Yellow and 5 mM ATP for 5 min, washed, and incubated in D10F at 37°C. At the indicated times, the coverslips were washed and examined by phase and fluorescence microscopy as described. (*a-c*) Immediately after ATP and Lucifer Yellow; (*d-f*) 30 min after ATP and Lucifer Yellow; (*g-i*) 60 min after ATP and Lucifer Yellow. (*a, d, and g*) Phase; (*b, e, and h*) fluorescein optics; (*c, f, and i*) rhodamine optics. In *a-c*, immediately after ATP permeabilization cells appeared more rounded than normal. Lucifer Yellow diffusely stained the cytoplasmic matrix of the J774 cells (*b*), while the Texas red ovalbumin-labeled lysosomes within the cytoplasm (*c*). 30 min after ATP permeabilization (*d-f*) the cytoplasmic matrix was nearly devoid of Lucifer Yellow, and the phase-lucent cytoplasmic vacuoles (shown in *d*) had taken up Lucifer Yellow (*e*). These vacuoles were distinct from the Texas red ovalbumin-stained lysosomes (compare *e* and *f*). 60 min after ATP permeabilization, Lucifer Yellow and Texas red ovalbumin exhibited nearly identical intracellular distributions (compare *h* and *i*). Bar, 20 μ m.

low was loaded into J774 cells, dye secretion and sequestration had taken place; most of the dye remaining inside the cells was present in phase-lucent vacuoles (Fig. 1, *d* and *e*).

J774 cells grown in bicarbonate-buffered medium contained few phase-lucent vacuoles immediately before or after exposure to ATP, but produced more of these vacuoles 10–20 min later, as described in Materials and Methods. Cells grown in Hepes-buffered medium possessed a greater number of phase-lucent vacuoles both before and after incubation in ATP and were used for illustrative purposes in Fig. 1, *a-c*. However, the processes of dye secretion and sequestration

described below were qualitatively similar in cells maintained in bicarbonate- and Hepes-buffered medium.

To explore the fate of the Lucifer Yellow sequestered in vacuoles and to determine the relationship of the sequestering vacuoles to lysosomes, we pre-labeled the lysosomal system of the cells with Texas red-labeled ovalbumin, loaded the cytoplasmic matrix of these cells with Lucifer Yellow by ATP permeabilization, washed the cells to remove ATP and Lucifer Yellow from the medium, and incubated the cells at 37°C. J774 cells incubated in Texas red-labeled ovalbumin for 1 h (Fig. 1 *i*) accumulated this labeled protein within

their distinctive system of tubular lysosomes (23). These lysosomes form an interconnected array of tubular structures that radiate from the centrosphere region in association with microtubules and exhibit saltatory motion (21, 22).

After cells whose lysosomes had been labeled with Texas red ovalbumin were loaded with Lucifer Yellow by ATP permeabilization, the Lucifer Yellow diffusely stained the nuclear and cytoplasmic matrices (Fig. 1 *b*). The network of Texas red-stained tubular lysosomes was disrupted by ATP treatment, and appeared as discrete vesicular and vermicular structures (Fig. 1 *c*). There was no evidence that Texas red was released into the cytoplasmic matrix. When these cells were further incubated for 30 min at 37°C, their phase-lucent vacuoles were filled with Lucifer Yellow (Fig. 1 *e*) but remained unstained by Texas red ovalbumin (Fig. 1 *f*), demonstrating that these vacuoles were distinct from preformed lysosomes. By this time, Texas red ovalbumin was seen again in interconnecting tubules that exhibited saltatory movement, indicating that the cells' lysosomal system had regained structural integrity.

In cells incubated at 37°C for 1 h after exposure to ATP, most cytoplasmic vesicles and tubules that were labeled with Texas red ovalbumin also contained Lucifer Yellow (cf. Fig. 1, *h* and *i*). The distribution of dye-containing organelles was characteristic of the lysosomal compartment of J774 cells. Only an occasional vacuole contained Lucifer Yellow but not Texas red ovalbumin.

These studies show that Lucifer Yellow introduced into the cytoplasm of J774 cells by ATP-permeabilization is transferred from the cytoplasm into a prelysosomal vacuolar compartment, and that these vacuoles subsequently deliver Lucifer Yellow to lysosomes.

The Lucifer Yellow-sequestering Vacuoles are Derived in Part From Plasma Membrane

To determine whether the vacuoles that sequester Lucifer Yellow contain plasma membrane constituents, we labeled the plasma membranes of J774 cells before loading their cytoplasm with Lucifer Yellow and examined the fate of the labeled membrane at intervals thereafter. J774 cells were incubated for 15 min with anti-mouse macrophage plasma membrane antibody, washed, and then incubated for an additional 15 min with 20 µg/ml Texas red-labeled protein A, all at 4°C. Cells viewed by fluorescence microscopy at this point demonstrated intense Texas red fluorescence at their plasma membrane. Control cells incubated with Texas red protein A alone did not fluoresce.

J774 cells pretreated with antibody and Texas red protein A at 4°C were then incubated with ATP and Lucifer Yellow for 5 min at 37°C, washed, and bathed in fresh medium. Immediately after Lucifer Yellow loading, the cells exhibited diffuse intracellular Lucifer Yellow fluorescence (Fig. 2 *a*), while the Texas red-labeled antibodies remained at the plasma membrane (Fig. 2 *b*). When these cells were incubated for 30 min at 37°C, Lucifer Yellow was seen only within cytoplasmic vacuoles (Fig. 2 *c*), and these vacuoles also displayed Texas red fluorescence (Fig. 2 *d*). Distinct highlighting of the vacuolar membrane by Texas red could be discerned in some vacuoles.

Fluorescent labeling of the plasma membrane by the above method might induce cross-linking of membrane proteins;

therefore, in other experiments the plasma membrane of J774 cells was labeled directly with Texas red. J774 cells in suspension were bathed in PBS (pH 8.0) containing 10 µg/ml Texas red for 5 min at 4°C. The cells were washed, allowed to adhere to glass coverslips for 5 min, permeabilized with ATP in the presence of Lucifer Yellow, and viewed by fluorescence microscopy as described. Again, immediately after Lucifer Yellow was introduced into the cytoplasmic matrix the plasma membrane was labeled with Texas red, while after 30 min at 37°C the vacuoles that sequestered Lucifer Yellow were also labeled with Texas red (not shown).

The sequestering vacuoles could also be filled by endocytosis from the external medium. J774 cells were incubated at 37°C for 5 min in medium containing ATP but not Lucifer Yellow, washed, and incubated in D10F for 5 min to allow closure of the ATP-induced membrane pores. The cells then were incubated for an additional 10 min at 37°C in D10F containing 0.5 mg/ml Lucifer Yellow, washed, and observed by phase and fluorescence microscopy. Most of the phase-lucent vacuoles contained Lucifer Yellow; no Lucifer Yellow was present in the cytoplasmic matrix (not shown).

These observations reveal that the Lucifer Yellow-sequestering vacuoles are part of the cells' endocytic pathway and therefore either form directly by invagination of plasma membrane, or gain plasma membrane constituents by fusion with vesicles derived from the plasma membrane. These prelysosomal vacuoles exhibit some of the characteristics of endosomes.

Vacuolar Sequestration Does Not Occur by Pinocytosis After ATP-loading

Because the sequestering vacuoles could be labeled by pinocytosis of extracellular dye, one could argue that the "sequestering" described above was merely due to pinocytosis of extracellular dye after incubation of cells with ATP and Lucifer Yellow. We examined this possibility by assessing vacuolar sequestration of Lucifer Yellow in cells washed continuously with fresh medium. J774 cells plated on coverslips were loaded with Lucifer Yellow by ATP permeabilization, and the coverslips were then inverted on top of cylindrical lucite chambers containing ports at opposite sides of the chamber. Medium was infused continuously into one port and removed from the other. Despite the continuous removal of extracellular dye, Lucifer Yellow was sequestered within cytoplasmic vacuoles in these cells. Therefore, vacuolar sequestration of Lucifer Yellow is not due to reuptake of dye from the extracellular medium.

Sequestration Occurs at 18°C

Because transfer of solute from endosomes to lysosomes is reduced at 18°C (8), we studied the transport of Lucifer Yellow at this temperature. Coverslips with adherent J774 cells were preincubated with 20 µg/ml Texas red ovalbumin for 1 h at 37°C, exposed to Lucifer Yellow and ATP at 37°C, washed, and transferred to an 18°C water bath. Cells were observed at intervals thereafter. Sequestration of Lucifer Yellow into phase-lucent vacuoles occurred at 18°C, albeit more slowly than at 37°C (20). Lucifer Yellow-containing vacuoles were apparent at 30 min, and the cytoplasmic matrix was nearly devoid of dye by 1 h. However, at 30 min, 1, and

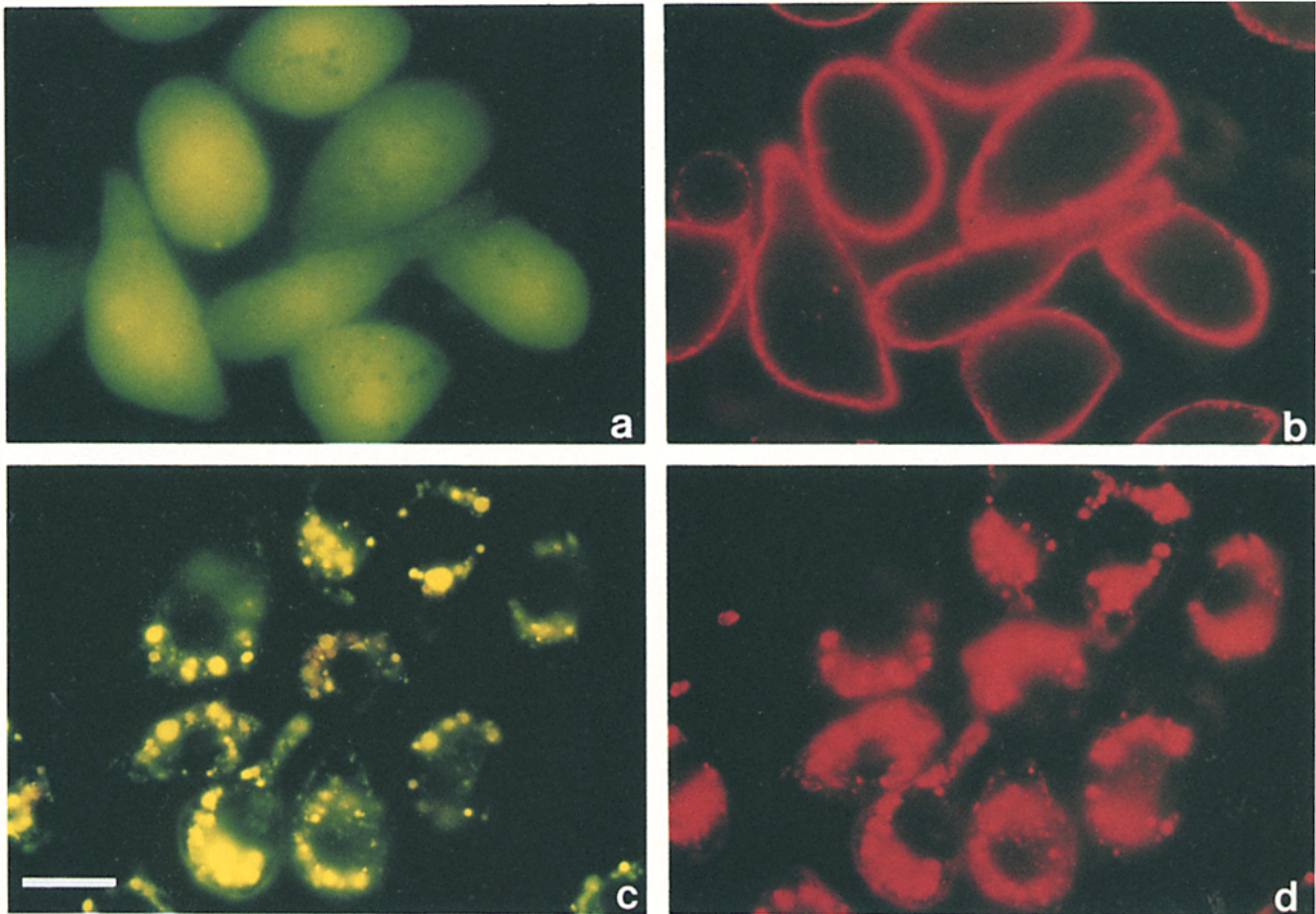


Figure 2. Vacuoles that sequester Lucifer Yellow contain plasma membrane-derived components. J774 cells adherent to glass coverslips were bathed in D10F containing anti-macrophage antibodies at 4°C for 15 min, washed, bathed in medium containing 20 µg/ml Texas red-conjugated protein A for 15 min at 4°C, washed, and incubated in D10F containing 5 mM ATP and 0.5 mg/ml Lucifer Yellow at 37°C for 5 min. Coverslips were washed, incubated in D10F at 37°C, and viewed by phase and fluorescence microscopy at the intervals indicated. (a and b) Immediately after exposure to ATP and Lucifer Yellow; (c and d) 30 min after exposure to ATP and Lucifer Yellow. (a and c) fluorescein optics; (b and d) rhodamine optics. Immediately after removal of ATP and Lucifer Yellow, Lucifer Yellow diffusely stained the cytoplasmic matrix and Texas red-conjugated protein A highlighted the plasma membrane of the cells. 30 min later, the vacuoles that had sequestered Lucifer Yellow from the cytoplasmic matrix also contained Texas red (compare c and d), indicating that the sequestering vacuoles contained plasma membrane-derived proteins. Bar, 20 µm.

2 h, no Lucifer Yellow was seen in the Texas red-labeled lysosomes, and the vacuoles that sequestered Lucifer Yellow did not contain Texas red. By 3 h, there was significant colocalization of the two dyes, and by 5 h, only a few vacuoles contained only Lucifer Yellow. These data show that at 18°C transfer of Lucifer Yellow from sequestering vacuoles to lysosomes was substantially slowed, consistent with our hypothesis that these vacuoles represent an endosomal compartment.

Ultrastructure of Sequestering Vacuoles After Treatment with ATP

Endosomes can be distinguished from lysosomes by electron microscopy. We therefore examined the ultrastructure of the phase-lucent sequestering vacuoles that formed after J774 cells were exposed to 5 mM ATP for 5 min at 37°C, washed, and incubated in D10F for 15 min at 37°C. The large cytoplasmic vacuoles seen at this time were bounded by a single

membrane, and had electron lucent interiors that only rarely contained internalized membrane (Fig. 3 a). The limiting membrane of some vacuoles exhibited evaginations (Fig. 3 b) resembling those observed in association with endosomes (12).

Lucifer Yellow Is Sequestered Initially Within a Vesicular Compartment of Low Buoyant Density and Later Within a Compartment of Greater Buoyant Density

Transport of endocytic markers from endosomes to lysosomes is reflected by an increase in the buoyant density of the membrane-bound compartment containing these markers. We therefore determined the buoyant density of Lucifer Yellow-containing organelles 15 or 60 min after loading the dye into the cytoplasm of intact cells. J774 cells adherent to 100-mm dishes were loaded with Lucifer Yellow by ATP permeabilization, washed, and incubated in fresh medium for

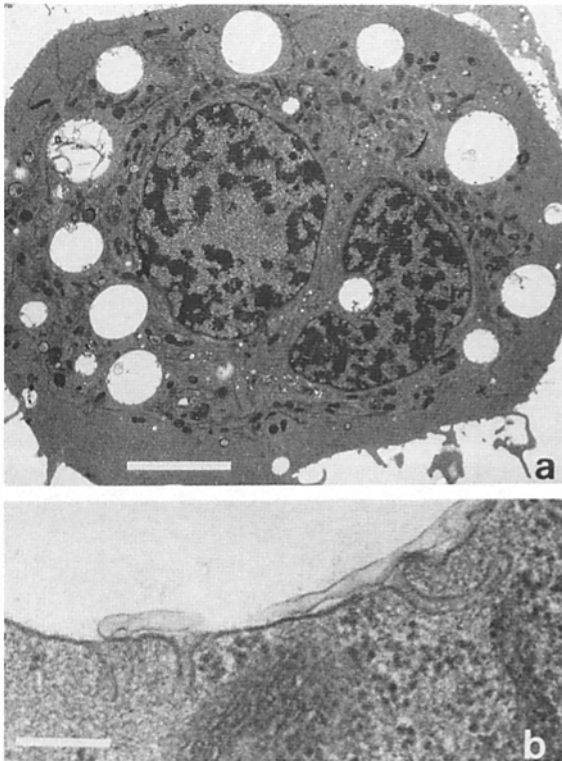


Figure 3. Ultrastructure of J774 cells 15 min after exposure to ATP. Cells were plated in 16-mm tissue culture wells, incubated in medium containing 5 mM ATP for 5 min at 37°C, washed, and incubated in D10F for an additional 15 min. Cells were then fixed and sectioned parallel to the plane of the culture dish, and viewed by transmission electron microscopy. (a) The phase-lucent vacuoles were bounded by a single membrane and were devoid of electron dense contents. (b) Evaginations from the vacuolar membrane. Bars: (a) 5 μ m; (b) 200 nm.

15 or 60 min. The cells were harvested and homogenized, and a large particulate cytoplasmic fraction was obtained from the post-nuclear supernatant by centrifugation. Vesicles from this fraction contained Lucifer Yellow when examined by fluorescence microscopy. The large particulate cytoplasmic fraction was spun through a percoll density gradient, and the membranes from gradient fractions were assayed for Lucifer Yellow content, alkaline phosphodiesterase, and beta-glucuronidase.

The large particulate fraction of J774 cells reliably formed two visible bands on percoll gradients. Fractions 1 and 2 (from the bottom of the gradient) contained membrane-bound granules with electron dense contents, as determined by transmission electron microscopy (not shown). These fractions also contained most of the beta-glucuronidase activity (Fig. 4, top), and therefore represent the lysosomal fraction. The band of lower buoyant density was located in fractions 8 and 9 near the top of the percoll gradient, and was coincident with most of the alkaline phosphodiesterase I activity. These fractions therefore contained plasma membrane and, as demonstrated by Galloway et al. (11), are expected to contain early endocytic vesicles including endosomes. The distributions of beta-glucuronidase and alkaline phosphodiesterase I on these gradients were not affected by the

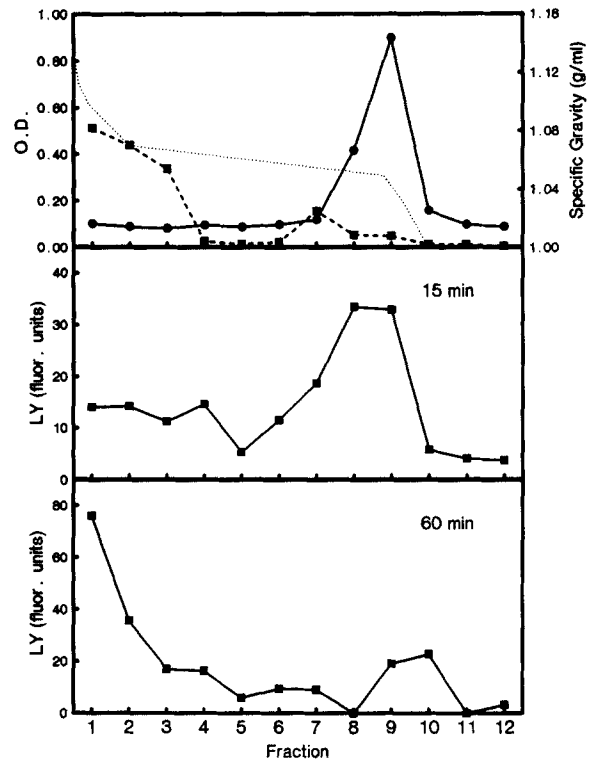


Figure 4. Lucifer Yellow is sequestered initially within a vesicular fraction of light buoyant density and later is found in a vesicular fraction of greater buoyant density. $3-4 \times 10^7$ J774 cells adherent to 100-mm tissue culture plates were loaded with Lucifer Yellow, washed, incubated at 37°C for 15 or 60 min, and homogenized. 2 ml of the large particulate cytoplasmic fractions were layered on 10 ml percoll gradients and centrifuged, and 1-ml fractions were collected from the bottom of the gradients. These fractions were assayed for alkaline phosphodiesterase I, beta-glucuronidase, and Lucifer Yellow content. See Materials and Methods for details. The top panel shows the peak concentration of alkaline phosphodiesterase I (solid line), which corresponded to the upper band of the percoll gradient, and the peak concentration of beta-glucuronidase, (broken line) which corresponded to the lower band. The dotted line depicts the specific gravity of the gradient fractions. The percoll density gradient distribution of vacuoles containing Lucifer Yellow after sequestration for 15 or 60 min are shown in the middle and lower panels, respectively. Fractions are numbered from the bottom of the gradient.

length of time the cells were incubated after ATP treatment or by the presence of Lucifer Yellow.

The distribution of Lucifer Yellow in the large particulate fraction from cells allowed to sequester Lucifer Yellow for 15 min before fractionation is depicted in the middle panel of Fig. 4. Most of the membrane-associated dye from these cells was present in gradient fractions 8 and 9, the fractions which contained most of the alkaline phosphodiesterase I. However, in cells which had been incubated at 37°C for 60 min after Lucifer Yellow was introduced into the cytoplasm (Fig. 4, bottom), most of the dye was found in the gradient fractions 1 and 2, those containing the lysosomal enzyme beta-glucuronidase. These fractionation experiments confirm the data obtained by fluorescence microscopy: Lu-

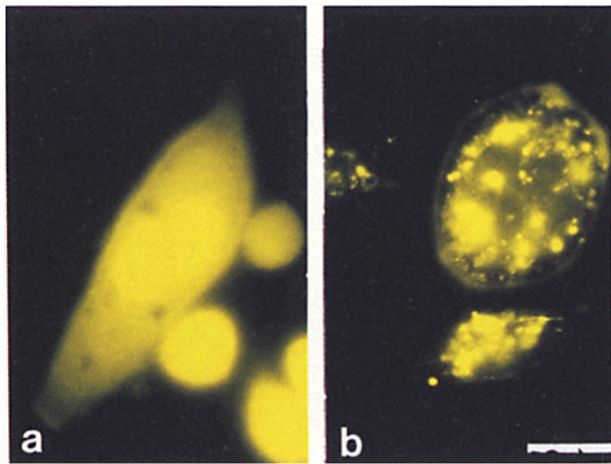


Figure 5. Lucifer Yellow sequestration within cytoplasmic vacuoles in J774 cells is inhibited by probenecid and resumes upon removal of probenecid from the medium. Adherent cells were incubated in D10F containing 5 mM ATP and 0.5 mg/ml Lucifer Yellow at 37°C for 5 min, washed, and bathed at 37°C in D10F containing 5 mM probenecid. After 30 min, some samples were washed and incubated in D10F without probenecid. Cells were viewed by fluorescence microscopy 1 h after removal of ATP and Lucifer Yellow. (a) 5 mM probenecid throughout; (b) 5 mM probenecid for 30 min followed by D10F alone for 30 min. Bar, 20 μ m.

cifer Yellow initially was sequestered in vacuoles which had the sedimentation properties of endosomes, and later appeared in the fractions with the density of lysosomes.

Probenecid Inhibits Sequestration of Lucifer Yellow Within Cytoplasmic Vacuoles in J774 Cells

Secretion of Lucifer Yellow from J774 cells and mouse peritoneal macrophages is inhibited by the drug probenecid (20), which blocks the transport of organic anions across many epithelia. To determine whether the sequestration of Lucifer Yellow into cytoplasmic vacuoles also is mediated by probenecid-inhibitable transporters, J774 cells were loaded with Lucifer Yellow in the presence of probenecid, washed to remove extracellular dye, and further incubated at 37°C for 20 to 60 min and examined by fluorescence microscopy (Fig. 5 a). Probenecid treatment blocked Lucifer Yellow efflux, and the cells' nucleoplasm and cytoplasm remained diffusely stained with this dye as reported previously (20). Phase-lucent vacuoles in these cells appeared to exclude Lucifer Yellow, but the diffuse cytoplasmic fluorescence made it difficult to determine this with certainty.

To verify that probenecid inhibited vacuolar sequestration, cells were loaded with Lucifer Yellow, incubated for 20 min in the presence or absence of probenecid, and then permeabilized with antibody and complement. This treatment released Lucifer Yellow from the cytoplasmic matrix and allowed us to assess the presence or absence of Lucifer Yellow-filled vacuoles in probenecid-treated cells. Cells treated with probenecid contained little intravacuolar Lucifer Yellow, while cells that were not treated with probenecid possessed numerous Lucifer Yellow-filled vacuoles (Fig. 6). Because inhibition of organic anion transport almost totally

prevented the appearance of Lucifer Yellow in cytoplasmic vacuoles, this experiment also provided additional evidence that the dye did not enter vacuoles by pinocytosis.

The inhibition of Lucifer Yellow sequestration by probenecid was reversible. Cells were loaded with Lucifer Yellow and maintained in medium containing probenecid for 30 min, washed, and incubated for an additional 30 min in medium without probenecid. Lucifer Yellow was sequestered within vacuoles in cells viewed at this time (Fig. 5 b).

To demonstrate that probenecid did not affect the formation of phase-lucent vacuoles that sequester Lucifer Yellow, we compared the number of these vacuoles in probenecid-treated and control J774 cells 15 min after their exposure to ATP. The probenecid-treated cells contained 570 vacuoles per 100 cells (average of 5 replicate cultures), while the control cells contained 548 vacuoles per 100 cells (average of 5 replicate cultures). This difference was not significant.

We derive two major conclusions from these experiments. First, sequestration of Lucifer Yellow into vacuoles was mediated by organic anion transporters as is secretion of Lucifer Yellow from the cells. Second, vacuole formation was not dependent on the entry of anions into this compartment via probenecid-sensitive transporters.

Lucifer Yellow sequestration was not dependent on acidification of the vacuoles. When 10 μ M monensin or 300 μ M chloroquine was added during ATP permeabilization and the subsequent 30 min incubation at 37°C, sequestration of Lucifer Yellow was not inhibited, although chloroquine markedly increased the size of intracytoplasmic vacuoles. Valinomycin (10 μ M) or valinomycin and nigericin (1 μ M) similarly did not block dye sequestration. Preincubation of cells with 20 mM 3-methyladenine, which inhibits autophagy in rat hepatocytes (15) also did not disrupt sequestration of Lucifer Yellow.

Discussion

In these studies a prelysosomal vacuolar compartment sequestered Lucifer Yellow from the cytoplasmic matrix of J774 cells and subsequently delivered this dye to lysosomes. These dye-sequestering vacuoles have several characteristics of endosomes: they received endocytosed material within a few minutes, and delivered their contents to lysosomes; transfer of dye from these vacuoles to lysosomes was slowed markedly at 18°C; and they had a buoyant density similar to that of endosomes.

Morphologic evidence indicated that Lucifer Yellow was transferred from these phase-lucent vacuoles to tubular lysosomes. Moreover, in studies of living cells by fluorescence microscopy we have observed fusion between Lucifer Yellow-containing vacuoles and tubular lysosomes. That these vacuoles are distinct from pre-formed lysosomes is supported by our observation that the vacuoles are unstained in cells whose lysosomal compartments were labeled with Texas red-labeled ovalbumin before treatment with ATP (Fig. 1). Ultrastructural studies showed that the sequestering vacuoles are surrounded by a single membrane, and lack electron opaque contents. The latter characteristic distinguishes them from the bulk of secondary lysosomes found in these cells. Subcellular fractionation provided independent confirmation that the sequestering vacuoles were distinct from lyso-

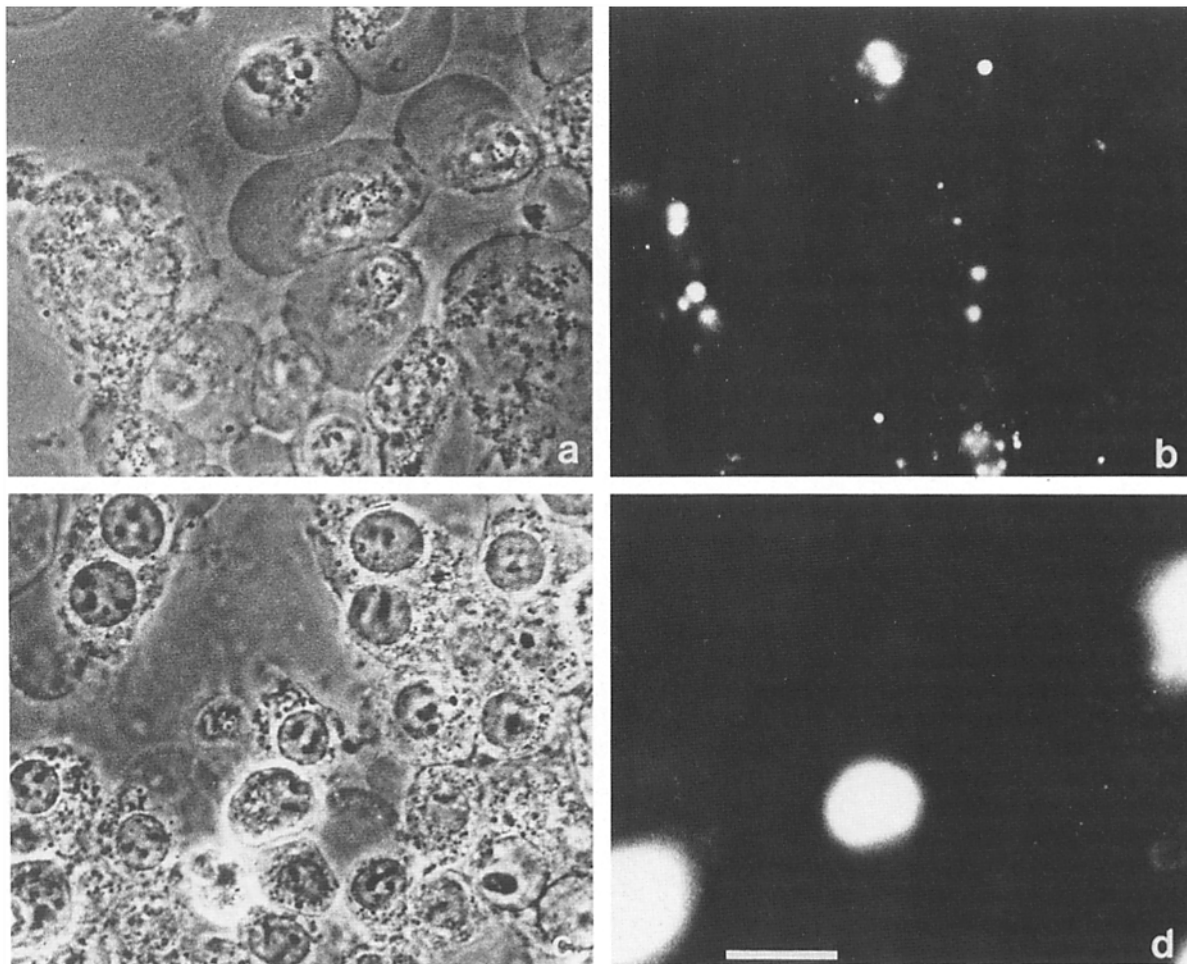


Figure 6. Sequestration of Lucifer Yellow is inhibited by probenecid and is not due to pinocytosis of dye. Cells adherent to glass coverslips were loaded with Lucifer Yellow in the presence or absence of 5 mM probenecid, washed, incubated in DME containing anti-macrophage antibody for 10 min at 37°C, washed, and incubated for 10 min in DME containing 20% fresh human serum as a source of complement. This treatment permeabilized the plasma membranes of most cells, resulting in release of cytoplasmic dye. In the absence of probenecid (*a* and *b*) cytoplasmic vacuoles sequestered the dye during the 20-min incubation at 37°C. In cells treated with probenecid (*c* and *d*) no vacuoles containing Lucifer Yellow were present. In the presence of probenecid, the few cells that were not permeabilized by antibody and complement remained diffusely fluorescent. Bar, 20 μ m.

somes: in cells fractionated 15 min after Lucifer Yellow was introduced into the cytoplasm, the dye appeared predominantly in the membrane fractions of low buoyant density known to be enriched in endosomes; in cells fractionated after 60 min, Lucifer Yellow appeared predominantly in the lysosomal fractions of the gradient (Fig. 4).

Nevertheless, the sequestering vacuoles are part of the endocytic pathway. The phase-lucent Lucifer Yellow-sequestering vacuoles were intensely labeled in cells whose plasma membranes were tagged with Texas red or Texas red-labeled antibodies before ATP treatment (Fig. 2), and these vacuoles could be labeled with Lucifer Yellow by pinocytosis when Lucifer Yellow was added to the medium after ATP was removed. These characteristics distinguish the sequestering vacuoles from organelles derived from internal membranes.

Studies of the kinetics of formation of these vacuoles in response to ATP⁴⁻ treatment show that the vacuoles occur after ATP and Lucifer Yellow have been removed from the medium and the membrane pores induced by ATP have

closed. This observation provides additional evidence that sequestration is not a consequence of uptake of Lucifer Yellow from the extracellular fluid.

Most of the Lucifer Yellow introduced into the cytoplasmic matrix by ATP permeabilization is secreted into the extracellular medium within 30 min after ATP-permeabilization is reversed (20). Our studies define two routes by which this dye secretion could take place: direct secretion by transporters present in the plasma membrane, or sequestration into cytoplasmic vacuoles by transporters present in the limiting membrane of these organelles followed by exocytosis. Because both of these pathways entail transport of dye across a membrane by a probenecid-inhibitable transporter, we could not use probenecid to determine the pathway of secretion of dye from these cells. Although the relative contributions of these two pathways to Lucifer Yellow secretion is unclear, the rapidity of dye efflux suggests that the former mechanism is important. Nevertheless, previous work indicates that 80% of pinocytosed Lucifer Yellow is returned to

the medium by exocytosis (23). Since the Lucifer Yellow-sequestering vacuoles are indeed endosomes, they may participate in the efflux of dye from the cells as well as the transfer of dye to lysosomes. Thus the pathway described in the present work might constitute an important secretory mechanism for molecules that pass through the cytoplasmic matrix, such as the various arachidonate derivatives released by macrophages.

Although exposure of cells to ATP transiently increased the number of phase-lucent vacuoles, sequestration of dye within cytoplasmic vacuoles was not dependent on exposure of cells to ATP. When Lucifer Yellow is "scrape loaded" into J774 cells, it accumulates within phase-lucent vacuoles (20). In addition, when carboxyfluorescein (Swanson, J., unpublished observation) and fura-2 (6) are introduced into the cytoplasm of cells as membrane permeant esters, they are hydrolyzed to their membrane impermeant forms. In both instances sequestration within vacuoles ensues.

The route followed by Lucifer Yellow from the cytoplasmic matrix to endosomes may provide a clue to the pathways followed by other cytoplasmic constituents as they are transported from the cytoplasmic matrix to lysosomes or the cell surface. There are at least two other situations in which membrane impermeant molecules have been shown or inferred to have been transported from the cytoplasmic matrix to the lysosomes or to the external face of the plasma membrane. These are the autophagic removal of senescent proteins (7, 16) and the processing and presentation of the internal antigens of certain animal viruses (25).

Radiolabeled sucrose is transported from the cytoplasm of hepatocytes into autophagosomes and subsequently delivered to lysosomes (24). Autophagy of rhodamine-conjugated proteins that were microinjected into HeLa cells also has been reported (16). The vacuoles into which these proteins were first transported did not contain acid phosphatase, but they later fused with lysosomes. A mechanism by which cytosolic proteins may be targeted to lysosomes has been suggested by Dice et al. who have identified a pentapeptide in ribonuclease A which destines this protein for lysosomal degradation in serum-starved human fibroblasts (7).

Processing and presentation of viral antigens appears to require transport of proteins from the cytoplasmic matrix to the vacuolar system, and subsequently to the cell surface. Townsend et al. have shown that influenza A specific cytotoxic T lymphocytes recognize epitopes of influenza proteins which are not expressed on the surface of the virus or of the infected cells (25). These findings suggest that viral proteins or protein fragments must be able to cross intracellular membranes, and lead us to speculate that the vacuole defined by Lucifer Yellow sequestration may be the compartment in which the processing of this and other antigens takes place.

Although the relationship of the Lucifer Yellow-sequestering vacuole to the autophagic processes described above is speculative at present, it is clear that sequestration of Lucifer Yellow defines a compartment that resides at an important intracellular crossroads, receiving molecules from the cytoplasmic matrix, and delivering them to the lysosomal compartment and probably to the plasma membrane and the extracellular environment. Evidence that the mannose-6-phosphate receptor shuttles between endosomes and the Golgi complex (3), and that endocytosed transferrin and endogenously synthesized cholinesterase are found in the same exo-

cytic vesicle (10) suggest that the endocytic and secretory pathways intersect. These findings raise the possibility that the Lucifer Yellow-sequestering vacuole also might intersect with this route out of the cell.

A large number of substances are transported across epithelial surfaces by probenecid-inhibitable transporters (5). Among these substances are leukotriene C₄, glutathione, bilirubin, and lactate, all of which are secreted by macrophages. The macrophage organic anion transporters are likely to be the pathway by which these products are secreted by macrophages; the sequestration of these molecules from the cytoplasmic matrix therefore constitutes regulatory mechanisms for their subsequent secretion by these cells.

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