Cell Killing by Lysosomotropic Detergents

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ABSTRACT We have studied the mechanism by which lysosomotropic detergents kill baby hamster kidney cells. Lysosomotropic detergents are lysosomotropic amines (compounds with pK between 5 and 9, such as imidazole or morpholine) containing straight-chain hydrocarbon "tails" of 9–14 carbon atoms (Firestone, R. A., J. M. Pisano, and R. J. Bonney. 1979, *J. Med. Chem.*, 22:1130–1133). Using lucifer yellow CH as a specific fluorescent label for lysosomes, it was shown by light microscopy that *N*-dodecyl (C_{12})-imidazole acted rapidly to damage lysosomes, causing leakage of dye into the cytoplasm. This was followed at later times by vacuolization, blebbing of the plasma membrane, cell rounding, and cell death. ^{3}H-labeled C_{12}-imidazole rapidly diffused into cells where much of it was trapped in lysosomes as shown by its co-migration with lysosomes in Percoll gradients. Cells preincubated with C_{12}-imidazole released it slowly into C_{12}-imidazole-free media, permitting the cells to be killed by the preincubation dose. Cell killing by the lysosomotropic detergents exhibited strongly sigmoidal dose-response curves. The sensitivity of baby hamster kidney cells to killing by C_{12}-imidazole was density dependent, the cells being most sensitive at lowest cell densities, and relatively resistant at confluence. The amount of ^{3}H-C_{12}-imidazole taken up by the cells was also density dependent, with highest specific uptake occurring at the lowest cell density. A rise in lysosomal pH, measured in fluoresceinated dextran-labeled cells, commenced immediately upon addition of C_{12}-imidazole to cells, and continued for over an hour. This was followed after a lag of 1–2 h by inhibition of protein and RNA synthesis and by lactate dehydrogenase release. Ionophores or lysosomotropic amines, such as methylamine, that raise intralysosomal pH provided substantial protection of the cells from killing by lysosomotropic detergents. These findings provide strong support for the idea that lysosomotropic detergents kill cells by disrupting lysosomes from within.

de Duve and his colleagues (12) applied the term "lysosomotropic" to various amines that share the property of becoming concentrated, often several hundredfold, within the lysosomal compartments of cells to which they are expressed. These compounds diffuse passively into the cellular lysosomal compartment where the low pH (4.7–4.8 in mouse peritoneal macrophages; 25) causes them to become protonated and trapped. It has now become clear that prelysosomal vesicles, or endosomes, also possess an acidic interior (33); hence, lysosomotropic compounds could accumulate in these vesicles by the same mechanism by which they accumulate in lysosomes.

Firestone et al. (16–18) have synthesized several compounds designed to combine lysosomotropism with detergent activity. By placing an amine of intermediate pK, such as imidazole or morpholine, onto a long hydrocarbon chain, a compound was produced that should diffuse across membranes, acquire a change by protonation in acidic compartments, and become trapped and accumulate in those same compartments. It was reasoned that when accumulation of the protonated form of the compound progressed to a certain stage, the material would develop surfactant properties and disrupt the lysosomal membrane, causing cell death by release of the lysosomal contents into the cytoplasm. Several lines of

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evidence suggested that the compounds synthesized to test these ideas did in fact act as "lysosomotropic detergents:" (*a*) The compounds were cytotoxic to several cultured cells at low concentrations but did not disrupt cells lacking lysosomes, such as erythrocytes; (*b*) some of the toxic compounds with low pK could not be protonated anywhere in the cell except lysosomes and endosomes; (*c*) intracellular vacuolization occurred prior to cell destruction; (*d*) the cytotoxic activity required a hydrocarbon chain of appropriate length, generally 12 carbons, while shorter chain homologs were less effective or inactive; (*e*) the dose dependence of cytotoxicity was sigmoidal and occurred over a narrow concentration range. This suggested that the cytotoxic action was cooperative, requiring interaction between several of the effector molecules, as would be required to form detergent micelles.

We have now studied the action of a lysosomotropic detergent, dodecyl (C_{12})-imidazole, on baby hamster kidney (BHK)[1] cells and their lysosomes in some detail. Our observations provide strong confirmation for the idea that disruption of the lysosomal membranes by trapped lysosomotropic detergent represents the underlying cause of the cytotoxic actions of these compounds.

MATERIALS AND METHODS

Cells and Reagents: BHK/21F cells obtained from the American Type Culture Collection (Rockville, MD) were maintained at 37°C in Dulbecco's minimal essential medium supplemented with 10% tryptose phosphate (Difco Laboratories, Detroit, MI) and 10% newborn calf serum (Gibco Laboratories, Grand Island, NY) in a humidified incubator with 5% CO_2/95% air. The cells were passed twice weekly in glass stock bottles and subcultured for experiments in Falcon or Costar plastic roller bottles, dishes, or clusters after removal from glass by trypsin/EDTA treatment. Cells used for experiments were grown for 1 or 2 d to 50–60% confluence unless otherwise indicated. Lysosomotropic detergents were prepared by the method described by Firestone et al. (16). ^{3}H-C_{12}-imidazole (0.22 μCi/μg) was prepared as described below. Percoll was obtained from Pharmacia, Inc. (Piscataway, NJ). All enzyme substrates and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO.)

2-^{3}H, 1-(n-$C_{12}H_{25}$) Imidazole: To 47 mg (0.2 mM) *N*-dodecyl imidazole in 2.5 ml of tetrahydrofuran was added 0.114 ml (0.2 mM) n-butyllithium at 0° under nitrogen. After 1.5 h, 0.020 ml of ^3H-H_2O (90 mCi/mmol) was added, followed by 5 ml each of ethylacetate and water. The aqueous layer was extracted once more with ethylacetate, and the combined ethylacetate layers were dried with $MgSO_4$, filtered, and evaporated. The residue, 40 mg, was chromatographed by thin layer chromatography on silica gel (20 × 20-cm plate, 0.5 mm thick) using 1:1 chloroform-ethylacetate, affording 27.8 mg of pure product, 0.22 mCi/mg.

Light and Fluorescence Microscopy: Cells were grown on either glass coverslips or Falcon 60-mm dishes with removable inserts (no. 3006; Falcon Labware, Oxnard, CA) for 1 to 2 d. Cells were labeled with lucifer yellow CH (LY, purchased from Sigma Chemical Co.) or fluoresceinated dextran (FD) (75,000 or 150,000 mol wt; Pharmacia, Inc. or Sigma Chemical Co.) overnight or with acridine orange (AO) for 15 min. Cells were washed at least five times in PBS and resuspended in serum-free medium without the fluorophore with or without lysosomotropic detergents and maintained until observation at 37°C. The glass slide or a 1-cm square piece of the Falcon plastic liner was then rinsed in warm PBS and inverted for viewing on a glass microscope slide with a drop of PBS. All photography was performed on a Nikon upright microscope with fluorescein isothiocyanate filters with a 40-power phase objective and 10-power eyepieces. High speed Ektachrome (Kodak, Rochester, NY) was used with a 25-s fluorescence exposure or a 5-s phase exposure. For quantitative purposes a Schoeffel RR 1000 spectrofluorometer as used to measure the fluorophores at the following wavelengths: (FD) 488-nm excitation, 519-nm emission; (LY) 448-nm excitation, 525-nm emission; (AO) 490-nm excitation, 534-nm emission.

Determination of Lysosomal pH in BHK Cell Monolayers: The procedure used was essentially that of Ohkuma and Poole (25). BHK cells were grown on glass coverslips and incubated with FD at a concentration of 0.5 mg/ml for 24 h or longer. The coverslip was washed five times with Earle's balanced salt solution, transferred to a fresh dish filled with medium, and returned to the incubator for at least 30 min. It was then washed ten times more and mounted diagonally in a standard 3-ml fluorescence cuvette. Fluorescence emission was detected at 90° from the incident beam. The spectrum of excitation from 450 to 490 nm was taken at room temperature, with emission set at 519 nm. The readings were corrected for blank cells lacking FD. The pH was determined from the 490:450 excitation ratio by comparison with a standard curve of FD in buffered solution.

Determination of Lysosomal pH Changes Induced by Lysosomotropic Detergents: BHK cells were grown on glass coverslips, labeled with FD, washed, and mounted as described above. The cuvettes containing the coverslips were equilibrated at 37°C, and were read (490-nm excitation, 519-nm emission) at periodic intervals after addition of the lysosomotropic detergent.

Subcellular Fractionation: BHK cells in 95% confluent roller bottles were washed three times with PBS, two times with Ca/Mg-free PBS, and two times with 0.25 M sucrose/1 mM EDTA, pH 6.8, all at 5°C. Cells were scraped with a rubber policeman in ~15 ml of the same sucrose/EDTA buffer, centrifuged at 1,000 rpm for 8 min in an International Equipment, Inc. centrifuge (Needham, MA), washed once more in sucrose/EDTA, and resuspended in the same at ~4 ml/roller bottle (~2 × 10^8 cells). Cells were broken open with 20 passes through a 10-ml disposable Corning pipette (75–90% breakage as judged by the percentage of free nuclei counted in a hemocytometer). If pipetting produced insufficient breakage, consecutive 15-s homogenizations with a Virtis 45 homogenizer (Virtis Co., Gardner, NY) at 50% maximum speed in a 5-ml glass homogenization chamber were used until ~90% breakage was achieved. With this fractionation technique excellent lysosomal latency was maintained, very little lysosomal enzyme activity being found in the cytosol lactate dehydrogenase (LDH) fractions (see Fig. 9). Nuclei and unbroken cells were removed by centrifugation at 2,000 rpm for 10 min. 3 ml of the resultant postnuclear supernatant was layered on top of gradients of Percoll (in the above sucrose/EDTA buffer), and the gradients were centrifuged as described in the figure legends.

Enzyme Analyses: LDH was analyzed by the method of Wroblewski and LaDue (35). Protein in the Percoll gradient was determined by the fluorescamine method of Udenfriend et al. (34) while all other protein determinations were determined by the method of Lowry et al. (22) using BSA as a standard after subtraction of appropriate Percoll blanks. Galactosyl transferase and 5′-nucleotidase were determined by the modifications of Rome et al. (30) of the procedures of Brew et al. (11) and Avruch and Wallach (6), respectively. β-*N*-acetylglucosaminidase was measured by a manual adaptation of the method of Beck and Tappel (9) using 3 mM *p*-nitrophenyl-*N*-acetyl β-D-glucosoaminide as a substrate with 1 N NaOH as the stopping reagent. Cytochrome c oxidase was measured by the technique of Appelmans et al. (5). NADPH-cytochrome c reductase was measured by the technique of Mahler (23).

Determination of Organic Phosphorus in Percoll Gradient Fractions: To 0.5 ml of each Percoll fraction was added 0.1 ml of 10% SDS in water, and the mixture was placed in a boiling water bath for 2 min. This was extracted with acidic chloroform-methanol as described by Bligh and Dyer (10). The sample was spun at 3,000 rpm for 15–20 min to compress the precipitated Percoll at the interphase, and the lower organic layer was carefully removed. This was evaporated to dryness in a boiling water bath, and total phosphorus measured by the method of Bartlett (7). 0.1 ml of 10 N H_2SO_4 was used for hydrolysis, followed by addition of sufficient H_2O_2 to oxidize all the carbon (generally a total of 0.3–0.6 ml was added in several aliquots over a period of hours while maintaining the sample at 150–160°C). The color development reaction described by Bartlett (7) was scaled down to 1 ml total volume and read at 830 nm in a Zeiss spectrophotometer.

RESULTS

Morphological Effects of C_{12}-Imidazole on BHK Cells. Specific Labeling of Lysosomes with LY CH

Direct observations of the effects of lysosomotropic detergents on the lysosomes of BHK cells were made by light microscopy of cells possessing fluorescently labeled lysosomes. Two fluorescent markers have been widely used to label lysosomes in living cells-acridine orange (AO) and fluoresceinated dextran (FD). AO is a weakly basic dye that enters

[1] *Abbreviations used in this paper:* AO, acridine orange; BHK, baby hamster kidney cells; FD, fluoresceinated dextran; LDH, lactate dehydrogenase; LY, lucifer yellow CH.

cells by diffusion (4; Miller, D., unpublished observations). It accumulates in nuclei in low concentrations (producing a dim green fluorescence) and in lysosomes in high concentrations (producing a bright orange fluorescence). Because AO diffuses across membranes, it is not possible to interpret a decrease in lysosomal fluorescence unequivocally as arising from disruption of the lysosomal membrane. FD is a polar fluorescent molecule that enters the cell exclusively by endocytosis. It accumulates in lysosomes, and has been successfully used for visualization of lysosomes and for measurement of intralysosomal pH in mouse peritoneal macrophages (25). In the case of BHK cells, we found that FD at subtoxic levels (below 0.5 mg/ml) labeled lysosomes to a barely observable intensity. This is not surprising, since the degree of substitution of commercial FD is only 1 fluorescein per 200 glucose monomer units. This procedure was therefore generally unsatisfactory, and led to a search for an improved method of specific lysosomal labeling in living cells.

Excellent lysosomal labeling was achieved using the fluorescent dye lucifer yellow CH (LY) (32). The following properties of LY made it advantageous for the present studies: (*a*) It is a pure fluorophore with a very high quantum yield, resulting in high, easily visualized fluorescence after uptake by cells; (*b*) it is nontoxic to cells, even at concentrations much higher than those required to label lysosomes; (*c*) it is highly hydrophilic, hence it does not diffuse across membranes but, like FD, enters cells exclusively by endocytosis or pinocytosis (data not shown); (*d*) it is small in molecular weight (457-mol wt), hence it will leak from lysosomes in response to minimal membrane damage; (*e*) it is resistant to bleaching, permitting extended viewing; (*f*) its quantum yield is independent of pH over a wide range.

Cells were labeled with LY or with AO to compare their intracellular localization. In Percoll gradients of the postnuclear supernatants of cells labeled for 18 h with LY, two-thirds of the LY fluorescence migrated with a dense lysosomal fraction. In contrast, in cells labeled for 45 min with AO (long enough for the maximal amount of AO to diffuse in), only one-fifth of the total AO was in lysosomes. The remainder co-migrated with light membranes and cytosolic enzymes (data not shown).

Fluorescence and phase contrast micrographs of untreated BHK cells labeled with LY are shown in Fig. 1, *a* and *b*. Lysosomes are clearly revealed; they are seen throughout the cell, but are concentrated in the perinuclear region. There is essentially no fluorescence outside of these discrete punctate regions, and the nuclear region appears completely black. In contrast, in AO-labeled cells (not shown) there was a pale green nuclear fluorescence and higher background fluorescence, which was consistently seen both intracellularly and, to a lesser extent, extracellularly.

When a cytotoxic amount of C_{12}-imidazole was added to BHK cells, e.g., 15 μg/ml (64 μM), the earliest morphological effect is a release of fluorescent marker from the lysosomes (Fig. 1, *c* and *d*) that gradually increases until the cytoplasm is extensively fluorescent (Fig. 1, *e* and *f*). This is best seen in LY-labeled cells at the area of the nucleus. Instead of being black, the region is marked by the diffuse yellow fluorescence that permeates the cytoplasm. In AO-labeled cells (not shown) a rapid release of dye from the vesicles was also observed. The fluorescent emission of the vesicles shifts from orange to yellow as the concentration of AO decreases. This decrease in AO fluorescence occurred much more rapidly than the leakage of LY from the lysosomes. In both AO- and LY-labeled cells, dye release occurs well before cell rounding (Fig. 1, *e* and *f*). Shortly after the onset of dye release, numerous vacuoles appear within the cell (Fig. 1, *g* and *h*; arrows). These vesicles do not accumulate LY or AO, and their origin is unknown.

Later stages of cytotoxicity are marked by increased clumping of the remaining lysosomes and cell rounding (Fig. 1, *g* and *h*) and the presence of blebs at the plasma membrane (not shown), observations similar to those in cells with lysosomes damaged by photosensitization (3). These blebs can be seen to be filled with cytoplasmic LY or AO. Even when cell rounding is well advanced, there remain some enlarged lysosomes that contain an appreciable amount of dye (Fig. 1, *g* and *i*). The cytoplasm becomes highly vacuolated. As the cells die, most of the LY leaks out; Fig. 1 *i* required an exposure three times as long as that for the other photographs. LDH is also released into the medium at the stage shown in Fig. 1, *i* and *j*.

The effect of C_{12}-imidazole at any time was generally not uniform over a whole culture dish. In some areas, all of the cells are affected by the C_{12}-imidazole simultaneously (Fig. 1, *e* and *f*), while in other areas the effects differ in adjacent cells (Fig. 1 *g*). As discussed below, these differences may arise from local variations in cell density. The progression of stages of cytotoxicity, however, appeared to be similar in each cell.

Nuclear staining became increasingly pronounced in AO-labeled cells as cytotoxicity advanced (not shown). The nuclei and nucleoli became more clearly delineated from the rest of the cell as vesicular fluorescence decreases. When cytotoxicity is far advanced (e.g., as in Fig. 1 *i*), only the nuclei remained fluorescent in AO-labeled cells (data not shown). The nuclear changes leading to increased AO binding are not known.

Agents that accumulate in lysosomes and raise lysosomal pH, but lack detergent properties, exhibited strikingly different effects on cells. Methylamine (20 mM) causes intense vacuolization of LY-labeled cells (Fig. 1, *k* and *l*). Vacuolization was limited to the perinuclear region, while the small vesicles in the periphery disappeared. There appears to be almost a line of demarcation between the swollen vacuoles and the vacuole-free cytoplasm. Most importantly, methylamine did not induce a permeability change in LY-labeled lysosomes, as evidenced by the lack of detectable cytoplasmic fluorescence (Fig. 1 *k*; cf. Fig. 1, *c* and *e*).

The intense vacuolization induced by methylamine was also evident in AO-labeled cells (not shown). However, most of the AO leaked out of the vesicles, leaving only a faint fluorescence. This is expected, since methylamine raises lysosomal pH, thus decreasing the pH gradient that holds the diffusible dye within the lysosomes. Conclusions regarding lysosomal permeability changes cannot therefore be made using AO-labeled cells. AO alone was also observed to cause spontaneous aggregation of tiny lysosomes into larger ones, thus markedly altering lysosomal distribution even after 1-h incubation at 37°C. This is similar to chloroquine-induced lysosomal changes that have been observed in macrophages (15).

Dependence of Cytotoxicity on Time of Incubation, Concentration of Lysosomotropic Detergent, and Cell Density

The dependence of cytotoxicity (as measured by LDH release) on the time of exposure of cells to increasing C_{12}-imidazole concentrations is shown in Fig. 2. Cytotoxicity was

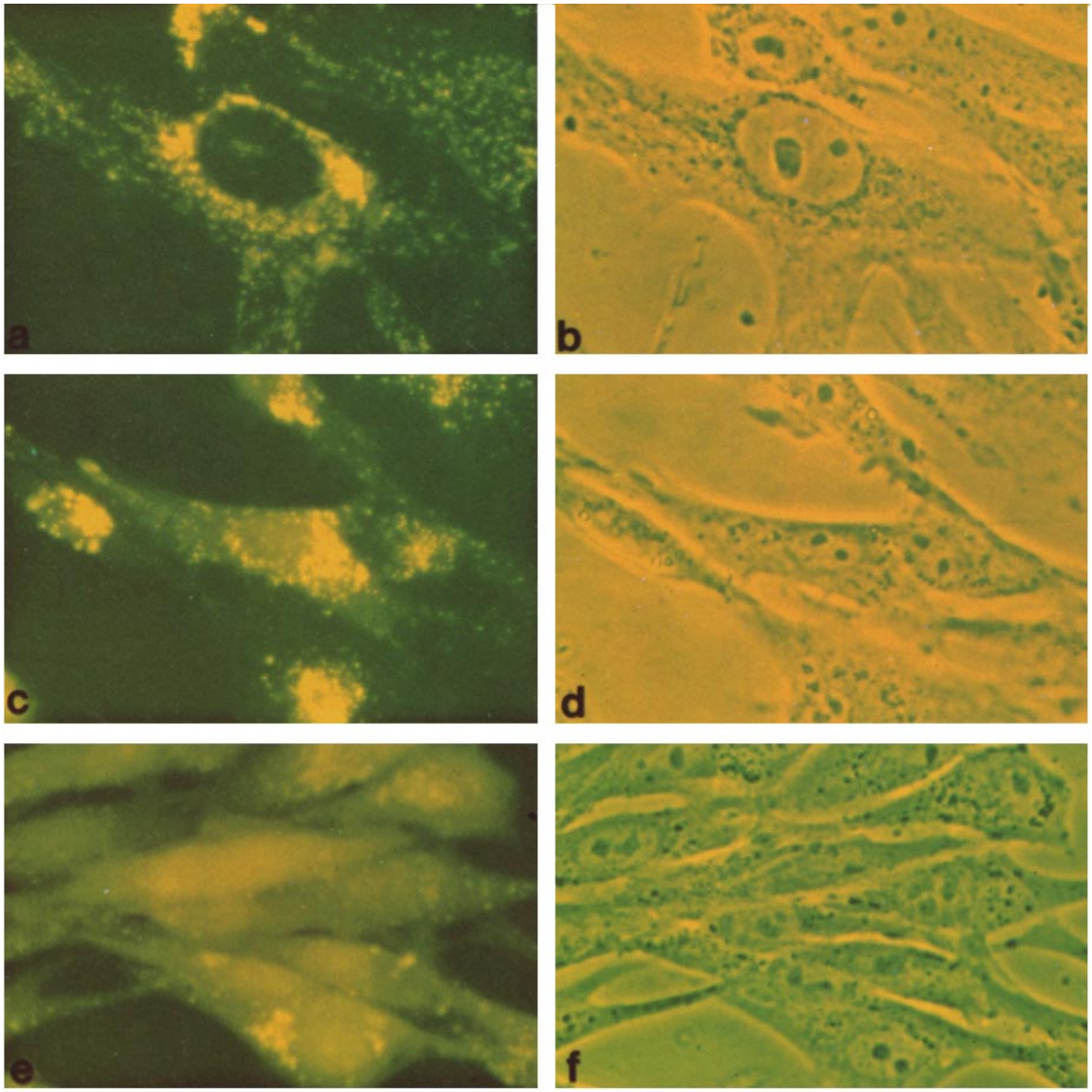
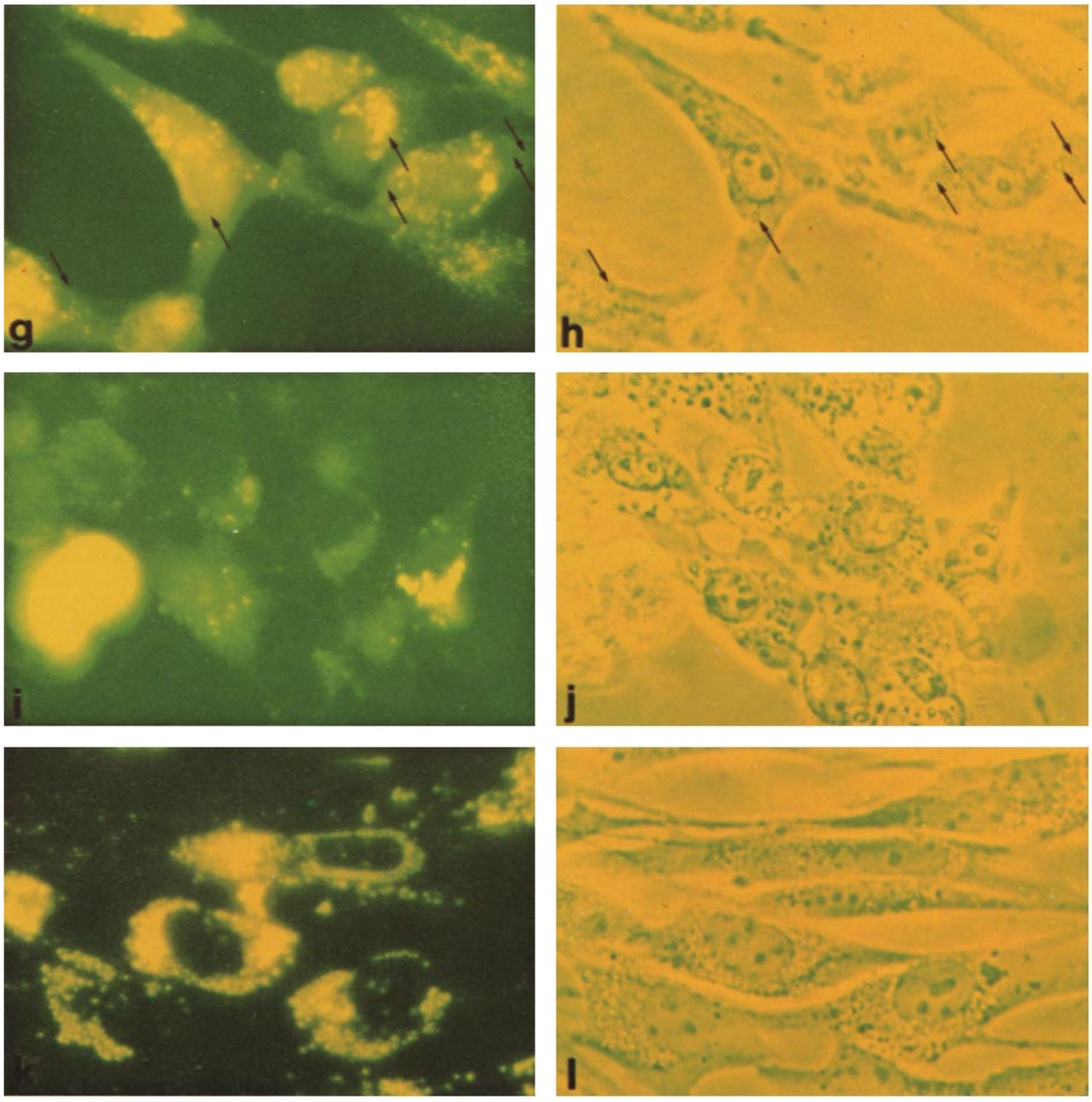


FIGURE 1 Fluorescence and phase micrographs of BHK cells labeled with lucifer yellow and treated with C_{12}-imidazole and methylamine. BHK cells were subcultured and grown for 2 d to 50% confluence on Falcon 60-mm dishes with removable plastic liners. LY was added to the medium at 1 mg/ml 16 h before observation. *a*, Fluorescence and *b*, phase micrographs of untreated cells; *c*, fluorescence and *d*, phase micrographs of cells treated with 15 μg/ml C_{12}-imidazole for 20 min; *e* and *f*, as in *c* and *d*, except that treatment was carried out for 30 min; *g*, and *h*, fluorescence and phase micrographs of cells treated with 32 μg/ml C_{12}-imidazole for 1 h; *i* and *j*, as in *g* and *h*, except that treatment was carried out for 2.5 h (fluorescence exposure was for 75 s, three times as long as the other fluorescence photographs); *k* and *l*, fluorescence and phase micrographs of cells treated with 20 mM methylamine for 45 min. Arrows in *g* and *h* indicate noncoincidence of C_{12}-imidazole-induced vacuoles (phase contrast, *h*) and LY stained vacuoles (*g*).

induced by C_{12}-imidazole in a concentration-dependent fashion during a 6-h incubation period. In this experiment, in which cells were used at ~25% confluence, the minimum concentration of amine required to give cytotoxicity within 6 h was 8 μg/ml (34 μM).

The sensitivity of cells to the C_{12}-imidazole was strongly density dependent. In an experiment comparing four different cell densities (ranging from 50% to confluence), it was clear that cells at the highest density were almost completely resistant to concentrations of C_{12}-imidazole that caused extensive killing in cells of lower density (Fig. 3). For example, at a concentration of 15 μg/ml (64 μM) of C_{12}-imidazole, the dense cells of Fig. 3*A* were almost unaffected by up to 4 h of exposure, while the lighter cells of Fig. 3*D* were almost totally killed. The toxicity of C_{12}-imidazole to each density of cells shows a similar inverse relationship between the time of exposure and the concentration of the amine (Fig. 4). But with increasing cell density the effectiveness of the amine decreases, shifting the toxicity curve to higher concentrations and/or longer exposure times (Fig. 4).

The cooperative nature of the lysosomotropic detergent effect is seen in Figs. 2 and 3. A distinctly sigmoidal dose-response curve is produced. This is consistent with a cooperative action of the C_{12}-imidazole molecules, suggesting that several molecules acting in concert, rather than individually, constitute the active cytotoxic agent (16, 17).



Entry of C_{12}-Imidazole into BHK Cells

C_{12}-imidazole entered cells rapidly, with uptake continuing for ~1 h before an equilibrium was reached, and no further uptake occurred (Fig. 5). The half-time of entry was ~10 min and was independent of concentration (data not shown). This rate was identical to that found for the uptake of AO and of methylamine in parallel experiments (data not shown). Both of these compounds enter cells by diffusion (12). In contrast, [^{14}C]sucrose, LY, and FD, compounds that enter cells by endocytosis, were found to enter BHK cells slowly, in a linear manner that continued for 1–2 d (data not shown). We conclude that C_{12}-imidazole, like AO and methylamine, enters cells by diffusion.

While the kinetics of entry of C_{12}-imidazole were similar at all cell densities, the amount of material taken up varied with cell density. The highest specific activity (expressed per gram of cell protein) is obtained at the lowest cell density (Fig. 5). The amount of C_{12}-imidazole that entered cells at any given cell density increased linearly with concentration, and showed no evidence of saturation (not shown). This is consistent with a diffusional mode of entry.

The decreasing sensitivity to the C_{12}-imidazole of the higher-confluence cells was not a consequence of insufficient detergent, as shown by two observations. First, even the most dense cells (confluence A) did not exhaust the supply of drug since they took up only 27% of the total. Confluences B and C, which were one-half and one-quarter as dense as A, took somewhat less, 23% and 20%, respectively. Confluence D, which showed early cytotoxicity, incorporated a maximum of 10% in 15 min (see Fig. 5). Second, similar variations in toxicity were observed on the same plate; i.e., cells in less confluent regions were affected earlier than those in more dense regions. Thus, even though the same concentration of C_{12}-imidazole was available to cells of differing density on a single plate, the less dense cells were more sensitive to the effects of the lysosomotropic detergents.

Reversibility of Interaction of Cells with Lysosomotropic Detergent

Lysosomotropic compounds are released only slowly from cells. BHK cells labeled with AO, for example, and incubated in medium without AO maintained 87% of the original amount within 0.5 h and 77% within 3.5 h at 37°C. In a similar experiment designed to test the reversibility of the action of lysosomotropic detergents, cells were incubated with two different concentrations of C_{12}-imidazole. When the cells were subsequently incubated in detergent-free medium, little if any C_{12}-imidazole leaked out of the cells (Fig. 6). The C_{12}-imidazole that remained in the cells was sufficient to kill the cells after a delay.

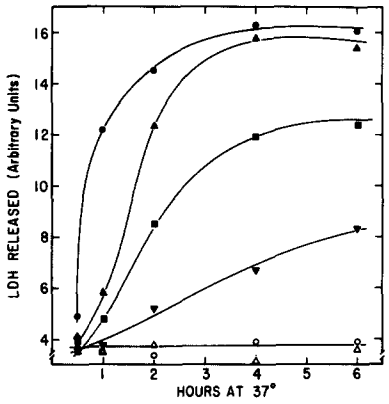


FIGURE 2 Toxicity of C_{12}-imidazole to BHK cells at various incubation times. Cells grown to ~25% confluence (8.8 μg/cm²) in 12-well Costar clusters were incubated for the indicated times at 37°C with various concentrations of C_{12}-imidazole (including 200,000 cpm ^{3}H-C_{12}-imidazole) in 0.5 ml of medium without serum. At the indicated times, the medium was removed and assayed for LDH content. C_{12}-imidazole concentrations were △, 4 μg/ml; 0, 6 μg/ml; ▼, 8 μg/ml; ■, 10 μg/ml; ▲, 12.5 μg/ml; ●, 15 μg/ml.

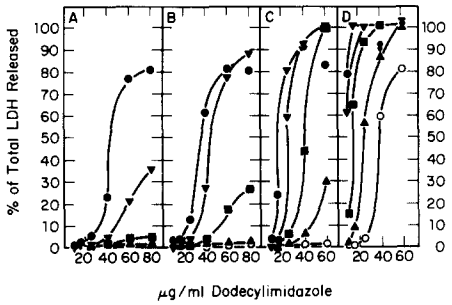


FIGURE 3 Toxicity of BHK cells exposed to increasing concentrations of C_{12}-imidazole at different exposure times and cell densities. Times of exposure were 0.25 h (○), 0.5 h (▲), 1 h (■), 2 h (▼), 4 h (●). Densities of cells (micrograms proteins per square centimeter) were *A*, 127 μg/cm²; *B*, 76.2 μg/cm²; *C*, 39.2 μg/cm²; *D*, 15.4 μg/cm².

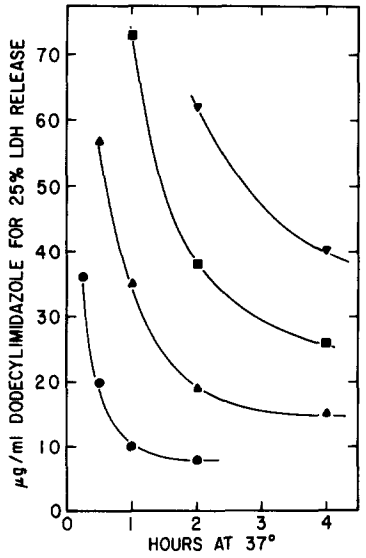


FIGURE 4 Relative toxicities to C_{12}-imidazole of BHK cells grown to different densities. Data were taken from Fig. 3. The concentration of C_{12}-imidazole needed to give 25% lactate dehydrogense released is plotted for each time of incubation. Density *A*, ▼; *B*, ■; *C*, ▲; *D*, ●.

Effects of Lysosomotropic Detergents on Lysosomal pH

Ohkuma and Poole (25) were the first to measure intralysosomal pH accurately in living cells. The ratio of fluorescence excitation of FD-labeled cells at two different wavelengths, 490 and 450 nm, provided a measure of intralysosomal pH that was independent of fluorescent intensity, and hence of the amount of intracellular FD. Changes in intralysosomal pH that occurred upon adding lysosomotropic amines could be followed by measuring the changes in excitation intensity at 490 nm with time. The excitation intensity of fluorescein at 490 nm increased about fivefold from pH 5 to 6, and about 15-fold from pH 5 to 7, providing a sensitive measure of changing lysosomal pH.

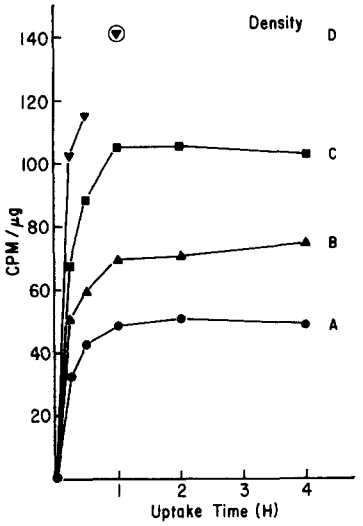


FIGURE 5 Time - dependent uptake of C_{12}-imidazole into BHK cells grown to different densities. C_{12}-imidazole was added at 15 μg/ml to cells with densities as shown in Fig. 3. The circled value at density *D* was from cells that showed amine-induced toxicity (some cells floating off the monolayer).

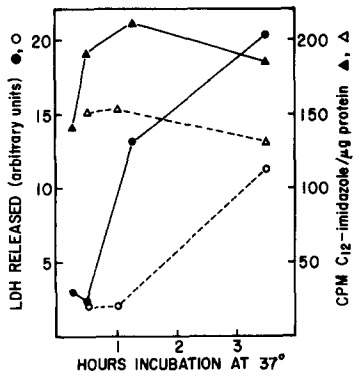


FIGURE 6 Washout of C_{12}-imidazole from BHK cells. Cells were incubated either (*a*) continuously for 3.5 h with ~50 μg/ml C_{12}-imidazole containing 10 μg/ml ^{3}H-C_{12}-imidazole. The medium was analyzed for lactate dehydrogenase content (●) and the cells for radioactivity (▲); or, (*b*) for 30 min with the same C_{12}-imidazole containing medium, washed, and then incubated in C_{12}-imidazole free medium for the indicated times. The medium was analyzed for lactate dehydrogenase content (○) and the cells for radioactivity (△).

We have used both types of measurement in this study. In an attempt to account for the density-dependent differences described above, we have used the 490/450 excitation method to measure the intralysosomal pH at several different cell densities. No density-dependent differences in lysosomal pH could be detected. The lysosomal pH, averaged over all readable cell densities (from ~30% to full confluence), was 5.07± .15 (average of 18 determinations).

We measured the changes in lysosomal pH induced by C_{12}-imidazole by measuring the change in fluorescence excitation at 490 nm of FD-labeled cells. Upon adding C_{12}-imidazole, the FD-measured pH rose gradually over a period of 1 h or more, depending upon the amount of detergent added (Fig. 7). This pH rise was not due solely to the neutralization of acid by the imidazole group, since methyl (C_1) imidazole at the highest equivalent molar concentration produced no change in lysosomal pH (Fig. 7). At the highest C_{12}-imidazole concentration tested (167 μM), the fluorescence excitation of FD at 490 nm increased about 16-fold, corresponding to a rise in pH from 5.0 to ca. 7.0–7.5. At both cytotoxic concentrations of C_{12}-imidazole shown in Fig. 7, LDH release lagged well behind the FD-measured pH rise, continuing to increase for at least an hour after the pH rise began to plateau. In contrast, addition of $(NH_4)_2SO_4$ (20 mM) caused a pH rise in <2 min, with no subsequent cell death. This rapid increase in pH agrees well with the findings of Ohkuma and Poole (25). The enhancement of excitation at 490 nm induced by $(NH_4)_2SO_4$ (about 8-fold) corresponds to a pH increase from 5.0 to ~6.1–6.5. Thus the slow, large rise in pH brought about by C_{12}-imidazole probably reflects porosity of the lysosomal membrane.

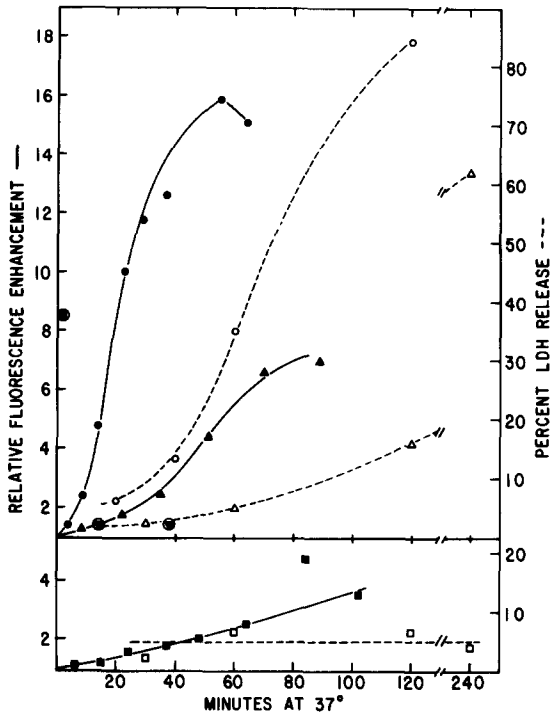


FIGURE 7 Effects of C_{12}-imidazole on lysosomal pH and cellular lactate dehydrogenase release. BHK cells were labeled with fluoresceinated dextran as described in Materials and Methods. Coverslips were incubated at 37°C with 40 μg/ml (167 μM) C_{12}-imidazole (●, ○), 20 μg/ml (▲, △), or 10 μg/ml (■, □). At the indicated incubation times, duplicate slides were used either to measure lysosomal pH changes (relative fluorescent enhancement at 490-nm excitation; see text) or lactate dehydrogenase release. Duplicate slides were also incubated with either 20 mM ammonium sulfate (⊙) or 167 μM methylimidazole (◒).

Effects of C_{12}-Imidazole on Protein and RNA Synthesis

Because LDH release is an end stage of cell cytotoxicity, it was necessary to determine what toxicity could be seen at earlier times. Protein synthesis is a sensitive indicator of overall cellular function, so the effect of C_{12}-imidazole on protein synthesis was compared with its effect on LDH release. As shown in Fig. 8, inhibition of protein synthesis was approximately coincident with LDH release. Inhibition of RNA synthesis was also found to coincide with LDH release (not shown). Thus the time interval between the initial effects on lysosomal pH and measured cytotoxic effects is quite long (Fig. 7) in contrast to the nearly coincident interval between expressions of the different cytotoxic effects (Fig. 8). This suggests that both the inhibition of macromolecular synthesis and cell killing are the consequence of a more fundamental traumatic event, one element of which may be the change in lysosomal permeability.

Intracellular Localization of C_{12}-Imidazole

To demonstate that C_{12}-imidazole actually concentrated in the acidic compartments of BHK cells, the cells were incubated with agents that raise intralysosomal pH for 30 min or 2 h prior to treatment with ^3H-C_{12}-imidazole. Table I shows that such treatment decreased the amount of lysosomotropic detergent taken up by the cells, regardless of whether the pH was raised by lysosomotropic amines such as methylamine or chloroquine, or by ionophores such as monensin or nigericin. Uptake was decreased by up to 38% at concentrations of amines or ionophores that are effective in raising lysosomal pH (25).

The intracellular distribution of ^3H-C_{12}-imidazole was determined by subcellular fractionation on Percoll density gradients (Figs. 9 and 10). The gradient in Fig. 9 was designed to

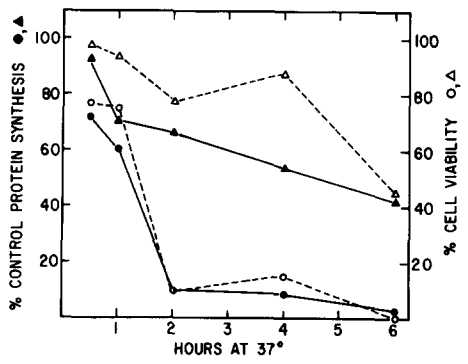


FIGURE 8 Effects of C_{12}-imidazole on protein synthesis and lactate dehydrogenase release. BHK cells were incubated with media containing 2 μCi/ml [^3H]leucine and either 16 μg/ml (△, ▲) or 32 μg/ml (○, ●), C_{12}-imidazole. At the indicated times, the medium was analyzed for lactate dehydrogenase activity. Cell viability is measured by the retention of intracellular lactate dehydrogenase (○, △) and the cells were washed five times, dissolved in 0.1% SDS, and precipitated in cold 5% trichloroacetic acid. The precipitated cell protein was filtered on Whatman GFC glass fiber filters, washed four times with 5% trichloroacetic acid, and counted for radioactivity (●, ▲).

TABLE I

Inhibition of Uptake of ^{3}H-C_{12}-Imidazole by Substances that Raise Lysosomal pH

Preincubation with	Concentration	Inhibition 0.5 h	Inhibition 2 h
		%	
Monensin	25 μM	23	35
Nigericin	1.25 μg/ml	26	36
Chloroquine	100 μM	16	27
Methylamine	20 mM	17	32

12-well cell clusters at ~ 50% confluence were incubated at 37°C with each agent for the indicated time before the addition of 1 μg/ml ^{3}H-C_{12}-imidazole (0.5 ml/well).

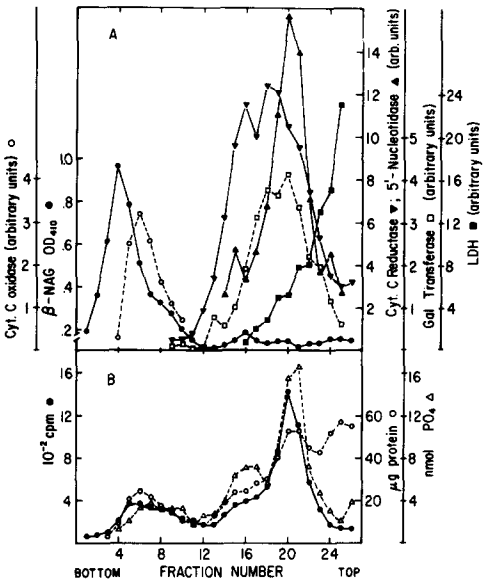


FIGURE 9 Percoll fractionation of postnuclear supernatants of BHK cells labeled for 1 h at 37°C with 4 μg/ml ^{3}H-C_{12}-imidazole. One roller bottle was incubated with 25 ml of the C_{12}-imidazole in serum-free medium. The postnuclear supernatant was layered over a Percoll gradient of 1 ml of 70% sucrose, 3 ml of 36% Percoll, 15 ml of 27% Percoll, 5 ml of 18% Percoll, and 13 ml of 4.5% Percoll and spun for 1 h at 18,000 rpm. Aliquots of the fractions were analyzed for enzyme markers, radioactivity, and protein and organic phosphorous. (*A*) O, cytochrome c oxidase (mitochondria); ●, β-*N* acetylglucosaminidase (lysosomes); ▼, cytochrome c reductase (endoplasmic reticulum); ▲, 5′ nucleotidase (plasma membrane); □, galactosyl transferase (Golgi); ■, lactate dehydrogenase; (*B*) ●, cpm; O, protein; Δ, organic phosphorous.

maximize separation of the lighter membrane components, hence the mitochondrial and lysosomal markers overlap. The distribution of ^{3}H-C_{12}-imidazole follows closely the distribution of phospholipid and noncytoplasmic cellular protein (Fig. 9*B*), with the protein, lipid and label all peaking around fraction 20, where the plasma membrane marker 5′-nucleotidase also peaks. A similar postnuclear supernatant was fractionated on a different Percoll gradient giving greater separation between lysosomes and mitochondria (Fig. 10*A*). When the ^{3}H-C_{12}-imidazole concentration was expressed relative to protein or to organic phosphorus, the specific activity was much higher in the lysosomal region of the gradient than in any other region (Fig. 10*B*).

The amount of radioactivity found in the lysosomal fractions varied considerably in different experiments. The highest amount found in lysosomal fractions was 27% of the total in the postnuclear supernatant; this was observed in an experiment in which the cell disruption procedure used was especially gentle and rapid. It seems likely, therefore, that variable amounts of labeled material were lost from the lysosomal fraction because of an increasing intralysosomal pH or leakage during and after mechanical disruption. Because of its hydrophobicity, the leaked lysosomotropic detergent would be expected to reequilibrate within the membranes of the homogenate where it is indeed found (Figs. 9 and 10). These results support the idea that the lysosomotropic detergent is concentrated in the lysosomal compartment inside the living cell.

Protection from Lysosomal Detergent Cytotoxicity by Raising Lysosomal pH

Since raising the intralysosomal pH decreases the uptake of lysosomotropic detergents (Table I) it might also be expected to decrease their cytotoxicity. As shown in Fig. 11, treatment of cells with 15 mM methylamine delayed the onset of cytotoxicity by C_{11}-imidazole, a compound possessing lysosomotropic detergent properties very similar to those of C_{12}-imidazole.

In more extensive experiments with C_9-imidazole, both ionophores and amines were found to inhibit cytotoxicity. In the presence of the ionophores, nearly twice the detergent concentration was required to achieve the same cytotoxic effect (Table II). These agents did not, however, significantly protect the cells from the action of C_{12}-imidazole or C_{12}-morpholine. This may be due to the higher uptake of the C_{12}-

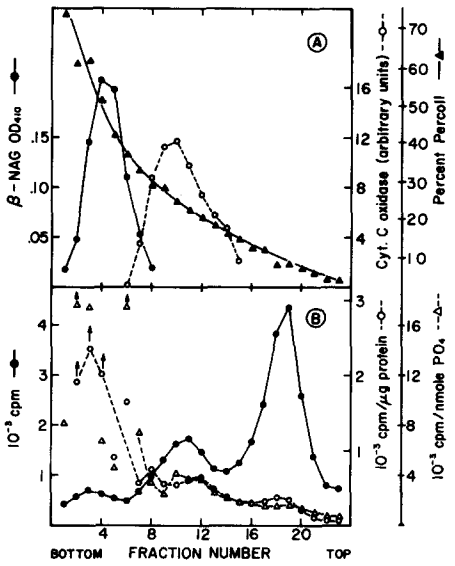


FIGURE 10 Percoll fractionation of postnuclear supernatants of BHK cells labeled for 1 h at 37°C with 1 μg/ml ^{3}H-C_{12}-imidazole. The postnuclear supernatant was layered on a Percoll gradient of 7 ml of 70% sucrose, 3 ml of 40% Percoll, 15 ml of 30% Percoll, 5 ml of 20% Percoll, and 13 ml of 5% Percoll and centrifuged as in Fig. 9. (*A*) ▲, density of a duplicate blank gradient in percent Percoll as determined by the light scatter measured in the spectrofluorometer set up to read LY fluorescence (see Materials and Methods); ●, β-*N*-acetylglucosaminidase; O, cytochrome c oxidase. (*B*) ●, counts per minute; O, counts per minute per gram protein, Δ, counts per minute per nanomole of organic phosphorous. High Percoll concentrations, and low protein and organic phosphorous levels, limited the accuracy of measurement in the lower fraction. Arrows above points indicate minimum values for specific activity.

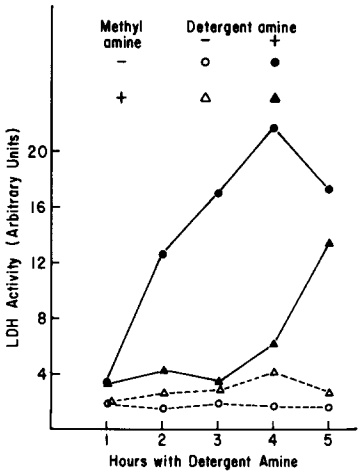


FIGURE 11 Prevention of C_{11}-imidazole induced toxicity in BHK cells by incubation with 15 mM methylamine. BHK cells preincubated for 30 min in medium with or without 15 mM methylamine were treated with ~15 μg/ml C_{11}-imidazole for the indicated times and the medium was analyzed for the lactate dehydrogenase released. Those cells preincubated with methylamine received methylamine for the remainder of the experiment.

TABLE II

Protection from Cell Killing by Substances that Raise Lysosomal pH

Lysosomotropic detergent	Protective amine	Time	Conc for 50% control	LDH released experimental	Control Experimental
		h			*μg/ml*
C_{12}-imidazole	$MeNH_2$	1	24.8	24.0	1.03
		3	17.8	20.0	0.89
	Nigericin	1	26.5	29.5	.90
		3	21.5	21.0	1.02
	Monensin	1	29.5	31.0	.95
		3	25.0	23.5	1.06
C_9-imidazole	$MeNH_2$	1	66	113	.58
		3	40	62	.64
	Nigericin	1	65	128	.51
		3	46	84	.55
	Monensin	1	71	130	.55
		3	48	58	.83
C_{12}-morpholine		1.75	83	77	1.08

BHK cells in 12-well clusters were preincubated for 2 h with either 2.5 mM methylamine, 1.25 μg/ml nigericin, or 25 μM monensin, followed by the addition of a lysosomotropic detergent at several concentrations: C_{12}-imidazole, 10–50 μg/ml; C_9-imidazole, 20–200 μg/ml; C_{12}-morpholine, 20–200 μg/ml. At 1 and 3 h after addition of lysosomotropic detergent, the concentration for 50% LDH release was determined for each lysosomotropic detergent. Control cells were treated with the same concentrations of lysosomotropic detergent, but neither the preincubation nor the incubation with lysosomotropic detergents contained a protective amine.

compounds (ca. 14-fold, not shown) as compared with the C_9 compounds as well as their greater detergency. In addition, it is well established that lysosomotropic detergents show narrow thresholds of activity, that is, the sigmoidal dose-response shows a high degree of cooperativity. The threshold concentration for weak detergents is higher than that for strong detergents. Consequently, it is reasonable that a given reduction in the uptake of C_9 imidazole may lower the intracellular concentration below the toxic threshold, whereas a similar reduction (Table I) in C_{12}-imidazole uptake leaves the intracellular concentration above the threshold.

Effects of C_{12}-Imidazole on Mitochondria

Subcellular fractionation studies showed that a significant amount of C_{12}-imidazole co-migrated with mitochondrial fraction (Figs. 9 and 10). The accumulation of C_{12}-imidazole in mitochondria is, however, unlikely to provide the basis of the cytotoxicity for several reasons. First, the specific activity was much lower in the mitochondrial fractions than in the lysosomal fractions. Second, inhibitors of oxidative phosphorylation (rotenone, 12.5 μM) and ATP-ADP exchange (atractyloside, 125 μM) had no effect on mitochondrial accumulation of ^3H-C_{12}-imidazole or on its cytotoxicity (data not shown). Both these agents shifted the mitocondria to lighter densities in the Percoll gradient (similar to prolonged incubation of cells at 50°C), indicating that these agents altered the physical state of the mitochondria. On the other hand, cytotoxic concentrations of C_{12}-imidazole had no effect on the position of the mitochondria in the gradient (data not shown).

DISCUSSION

The data in this report provide several new observations on the action of lysosomotropic detergents: (*a*) these compounds accumulate in lysosomes; (*b*) they alter the permeability of the lysosomal membranes, at least towards ions and small molecules; (*c*) these changes precede by a substantial length of time such direct cytotoxic effects on the cells as inhibition of protein synthesis, cellular rounding, and lactate dehydrogenase release; (*d*) agents that raise lysosomal pH give some measure of protection against the cytotoxic action of the detergents; (*e*) both cellular uptake and sensitivity to detergent amines is a function of the density and/or growth state of the cells, with the most rapidly growing cells being most sensitive. While the precise mechanism whereby lysosomotropic detergents kill cells is not yet known, it is highly likely from the above observations that it is the release of lysosomal enzymes into the cytoplasm that is the killing event, as suggested earlier by others (13, 12, 20). Such a release of lysosomal enzymes has indeed been demonstrated by other agents that damage lysosomes, including photosensitive dyes (3, 4), carcinogens (2), and viruses (1).

Because of the weakly basic character of lysosomotropic compounds, extensive accumulation inside lysosomes requires the operation of an energy-dependent proton pump (25, 31). But these compounds can also localize elsewhere in the cell to acidic components such as glycolipids (4). Thus, agents that deplete intracellular energy stores prevent accumulation only partially. We have observed this for lysosomotropic detergents (Table I), just as others have for methylamine (31). Consequently, it is not surprising that subcellular fractionation of cells incubated with lysosomotropic detergents should show only a minority of the material in the lysosomes (Figs. 9 and 10) and that this proportion should vary with the isolation procedure. What is significant regarding a possible lysosomal locus of action of the lysosomotropic detergents is (*a*) the high specific activity in lysosomes (Fig. 10*B*); and (*b*) the earlier observation that potential detergents that cannot be protonated at lysosomal pH (i.e., those with low pK) are not cytotoxic. Consider, for example, the toxicity of a series of lysosomotropic detergents reported earlier, namely $C_nH_{2n+1}NHCH_2CF_3$ (16). The C_{18} homologue with a pK of 4.7 was only one-fifth as active as the C_{12} homologue with a pK of 5.5. Yet the detergency of C_{18} and C_{12} chains are normally equivalent. In another example, $C_8F_{17}CH_2NH_2$ with a pK of 4.5 is poorly active (unpublished data), while the closely related compound $C_8F_{17}CH_2CH_2NH_2$, pK 8.3 (18) is a very powerful lysosomotropic detergent. Thus, the only locale in the cell where the class of lysosomotropic detergents

could have their effect is in lysosomes or another cellular locus of low pH.

The endosomes, or endocytic vacuoles, have recently been recognized as part of an important intracellular system that mediates transfer of materials between the cell surface and lysosomes, and likely between other intracellular organelles and even between cell surfaces. They sediment in Percoll gradients with light membranes, but lack specific marker enzymes. Endosomes are now known to have acidic interiors (33), and will therefore accumulate lysosomotropic detergents in the same manner as lysosomes. Although their membranes should therefore be disrupted in the same manner as those of lysosomes, a recent report suggests that disruption of endosomes is unlikely to be cytotoxic. Okada and Rechsteiner (26) recently reported that repeated disruption of intracellular endosomes by hypotonic treatment was without measurable cytotoxic effect, although substantial quantities of extracellular material was thereby introduced into the cytoplasm. Only lysosomes and endosomes are likely to have a low enough pH to protonate the amines used in this study, thus creating detergents. Consequently, the data of Okada and Rechsteiner are consistent with lysosomes rather than endosomes being the initial source of the toxicity of the lysosomotropic detergent.

We do not know why sparse, growing cells are much more sensitive than dense ones to the cytotoxic action of the detergent amines, but suggest three possibilities. (*a*) The lysosomal pH might rise with cell density, leading to a decrease in detergent amine accumulation, as observed. Our measurements of intralysosomal pH at different cell densities did not reveal any differences, although a pH difference as great as 0.3 U between highest and lowest densities might have gone undetected. This would correspond to a difference of about twofold in the amount of an amine accumulated, comparable with that observed (Fig. 5). (*b*) The lysosomal volume might decrease with increasing cell density as suggested by a cell-cycle-dependent change in total lysosomal volume of nearly 55% reported in hepatoma cells (8), paralleled by a change in the uptake of chloroquine (28). Although the precise relationship between cell density and cell cycle is not clear, these findings indicate that lysosomal volume, and thus uptake of amines, might vary with growth state. A decrease in lysosomal volume, if the concentrations of enzymes remained constant, would lead to a decrease in the amount of lysosomal enzymes available for cell killing. (*c*) The proton pump, like other energy-dependent metabolic processes, might slow down as cell density increases, resulting in a reduced response to the stress of proton leakage. This, in turn, would give rise to the observed reduction in uptake at high cell density.

Another class of cytotoxic agent that disrupts lysosomes is amino acid methyl esters. They enter isolated rat liver lysosomes by diffusion, are hydrolyzed, and accumulate as free amino acids, causing swelling and disruption of the lysosomes (19). They cause similar swelling of lysosomes in intact rat hearts (29, 21). However, disruption by osmotic swelling requires high concentrations (>50 mM) of leucine methyl ester in comparison with the osmotically insignificant amounts (<0.08 mM) of dodecylimidazole needed for comparable cytotoxicity under identical conditions in BHK cells (J. P. Reeves and D. K. Miller, unpublished observations).

Lysosomotropic detergents may prove useful in therapy where selective toxicity to lysosome-bearing cells relative to cells with fewer or no lysosomes is desired, for example in eliminating cancer cells from bone marrow during autologous transplantation. Dodecylmorpholine (120 μg/ml, 1 h) kills 92% of acute myeloid leukemia cells with no detectable loss of bone marrow cells (14). Dodecylmorpholine and *N*-dodecyl, *N*-(2,2-difluoroethyl)amine behave similarly with chronic myeloid leukemia vs. bone marrow cells (W. A. Robinson, unpublished experiments; 24). The compounds might also prove useful in selecting mutants defective in lysosomal acidification.

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