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Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents

(lysosomes/proton pump/ionophores)

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ABSTRACT **A quantitative method is described for the measurement of intralysosomal pH in living cells. Fluorescein isothiocyanate-labeled dextran (FD) is endocytized and accumulates in lysosomes where it remains without apparent degradation. The fluorescence spectrum of this compound changes with pH in the range 4–7 and is not seriously affected by FD concentration, ionic strength, or protein concentration. Living cells on coverslips are mounted in a spectrofluorometer cell and can be perfused with various media. The normal pH inside macrophage lysosomes seems to be 4.7–4.8, although it can drop transiently as low as 4.5. Exposure of the cells to various weak bases and to acidic potassium ionophores causes the pH to increase. The changes in pH are much more rapid than is the intralysosomal accumulation of the weak bases. Inhibitors of glycolysis (2-deoxyglucose) and of oxidative phosphorylation (cyanide or azide) added together, but not separately, cause the intralysosomal pH to increase. These results provide evidence for the existence of an active proton accumulation mechanism in the lysosomal membrane and support the theory of lysosomal accumulation of weak bases by proton trapping.**

There have been conflicting reports and theories about the pH inside lysosomes. This controversy has been the subject of a review by Tager and Reijngoud (1). Isolated lysosomes have an internal pH about 1 unit lower than that of the medium, apparently as a consequence of a Donnan equilibrium (2–4). However, there is some evidence of an energy-dependent mechanism that is capable of lowering the pH (5, 6). In living cells, various weakly basic substances are concentrated in lysosomes as a consequence of lysosomal acidity (7). When the intralysosomal concentration of these substances becomes sufficiently high, the lysosomes swell osmotically to form large vacuoles (8, 9). Vacuoles of similar appearance have been observed in cells exposed to the acidic ionophore X537A (10).

Attempts have been made to estimate the pH inside lysosomes by visual inspection of color changes in pH indicator dyes (see ref. 1). Here we describe a quantitative method for the measurement of intralysosomal pH based on the pH-dependent fluorescence signals from fluorescein isothiocyanate-labeled dextran (FD) in the lysosomes of living cells. Control experiments indicate that these signals should provide an accurate measure of pH. We report the results of some measurements of pH in the lysosomes of living cells under various conditions that support the existence of an active process of pH maintenance and provide some confirmation of the theory of pH-dependent concentration of certain substances in lysosomes (7).

MATERIALS AND METHODS

Mouse peritoneal macrophages isolated by the method of Cohn and Benson (11) were cultured in modified Eagle's medium (12) containing 20% fetal calf serum, gentamycin (25 μg/ml), and Fungizone (2.5 μg/ml) in glass Leighton tubes, either on the tube surface or on cover slips in an atmosphere of 5% CO_2 in air.

Fluorescence was measured with a Perkin–Elmer–Hitachi MPF-4 in ratio mode. Coverslips on which cells were growing were mounted in the holding device illustrated in Fig. 1. The holding device was inserted into a standard fluorescence cell and the cell was perfused with a thermostated solution containing the salts, glucose, and pyruvate of Eagle's medium plus various additions. All solutions were adjusted to pH 7.6. Serum and vitamins usually were omitted from the perfusion fluid because they interfered with the fluorescence measurements.

In uptake experiments, cells were exposed to the compound in question for various lengths of time and then washed four times with Hanks' solution (13). For the assay of chloroquine uptake, the cells were then dissolved in 0.1 M NaOH/0.4% sodium deoxycholate. For the assay of [^{14}C]methylamine uptake the cells were extracted twice with 4 ml of 5% trichloroacetic acid and then dissolved. Protein was measured by the automated Lowry procedure (14). Chloroquine was assayed fluorometrically. Scintillation counting was performed in Triton/toluene/2,5-diphenyloxazole.

FD (average molecular weight 67,000) was purchased from Sigma. Nigericin and X537A were generous gifts from Eli Lilly Co. and Hoffmann-La Roche Inc., respectively.

RESULTS

Fluorescence characteristics of FD

Fig. 2 shows the excitation spectra of solutions of FD at various pHs. The alkaline spectrum is dominated by a large peak at 495 nm. As the pH is lowered, this peak dies away and is replaced by two new, much lower, peaks at 480 and 450 nm. The intensity of the 495-nm peak could be used as a measure of pH, provided one knew exactly how much FD is being illuminated and provided the solvent remained the same. However, as shown in Fig. 3, a much better measure of pH is the ratio of fluorescence measured with excitation at 495 nm to that with excitation at 450 nm, to be referred to henceforth as the 495/450 ratio. We found that overall fluorescence intensity changes somewhat depending on the buffer used but the shape of the excitation spectrum remains almost constant at a given pH. The effects of ionic strength (Fig. 3) and bovine serum albumin (200 mg/ml) were small (<0.1 pH unit). Even at FD concentrations as high as 30 mg/ml, the fluorescence signals remained almost the same. In the work we describe below, we used the fluorescence intensity at 495 nm to follow rapid changes in intralysosomal pH and the 495/450 ratio to follow

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Abbreviation: FD, fluorescein isothiocyanate-labeled dextran.

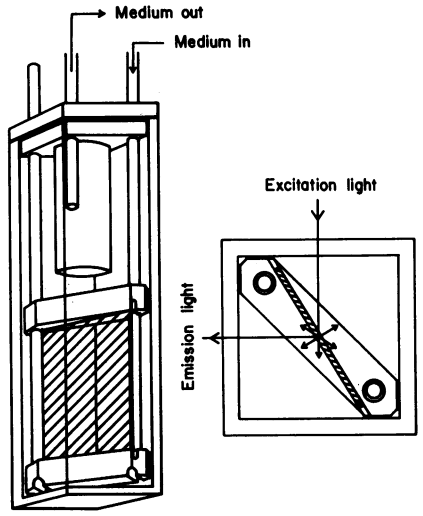

FIG. 1. Device used to hold coverslip in fluorescence cell in the spectrofluorometer. The coverslip (cross-hatched) is aligned at 30° to the excitation beam to minimize the effect of light reflections. Approximately 30 mm^2 of the coverslip is illuminated (about 10^5 cells).

slower changes when there was sufficient time to make a complete scan. As will be shown below, the two measurements agree fairly well.

Uptake and fluorescence of FD in lysosomes of macrophages

Macrophages exposed to FD in the medium (routinely, 1 mg/ml for 24 hr) take up about 6 μg of this compound per mg of protein. This FD remains in the cells in macromolecular form and can be visualized by fluorescence microscopy in a typical lysosomal pattern. After subcellular fractionation, the FD was found to be associated with marker enzymes for lysosomes. An excitation spectrum of FD within the lysosomes of living cells is shown in Fig. 4. The difference between the signals from cells with and without FD also is shown and is indistinguishable from a spectrum of FD at pH 4.75 calculated by interpolation of the data of Fig. 2.

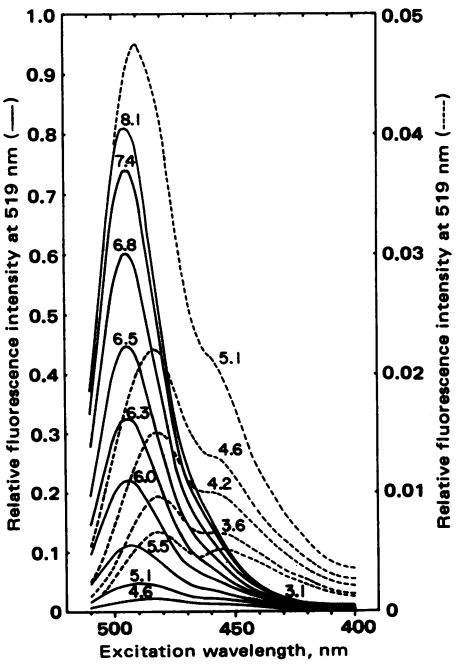

FIG. 2. Excitation spectra at 37° of FD at various pHs. All solutions at indicated pH contained FD at 1 μg/ml, 10 mM buffer (see legend to Fig. 3), and 50 mM NaCl. Emission was measured at 519 nm with 5-nm slits on both monochromators. The Raman spectrum of water (442 nm; maximum, 0.001 fluorescence unit) has been subtracted.

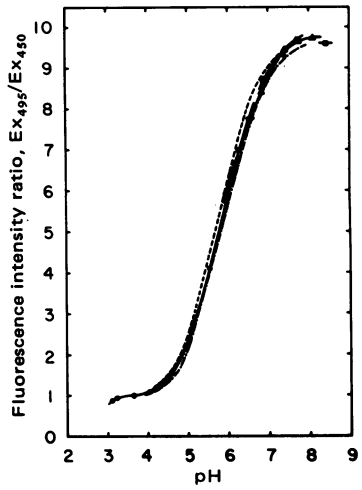

FIG. 3. FD 495/450 ratio (fluorescence with excitation at 495 and 450 nm) at various pHs; data from Fig. 2. Buffers (10 mM): ○, lactate; ●, acetate; △, phosphate; ▲, Tris; □, borate. – – –, No NaCl; - - - -, 100 mM NaCl.

We took cells out of complete medium containing 20% serum, washed them briefly in the salts, glucose, and pyruvate of Eagle's medium and then placed them in the coverslip holder in the perfusion chamber in the same solution. After such treatment the intralysosomal pH in a large number of experiments was always 4.5 ± 0.1. However, as illustrated in Fig. 5A, after further incubation in the chamber, the intralysosomal pH always increased to about 4.8 and then remained constant. When amino acids were included in the washing and perfusion medium, the intralysosomal pH remained about 4.9. When the perfusion medium was changed to one without amino acids, there was a transient decrease in the intralysosomal pH to about 4.5, with a gradual return to a stable value of 4.8 (Fig. 5*B*). As will be shown below, a similar transient low pH is observed when cells exposed to low concentrations of ammonia are washed with ammonia-free solution.

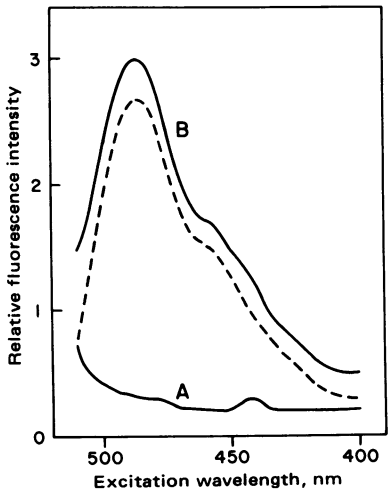

FIG. 4. Excitation spectra of macrophages before and after the endocytosis of FD. Curve A, control cells; curve B, cells containing FD. The dashed line is the difference between the two solid lines.

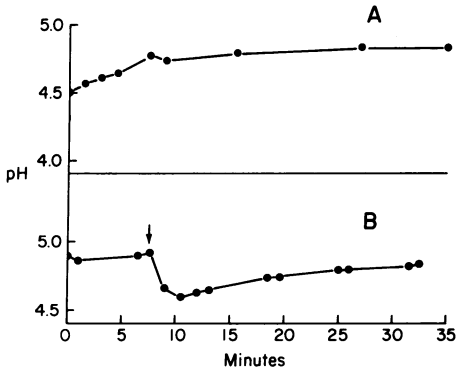

FIG. 5. (*A*) Coverslips bearing macrophages containing FD in their lysosomes were removed from complete medium containing serum, washed briefly, placed in a perfusion chamber (time 0), and perfused. (*B*) Coverslips were removed from complete medium and washed briefly as in A but wash solution contained amino acids. They were placed in the fluorescence cell, perfused with the same medium for 7 min, and then (arrow) perfused with wash solution. pH was calculated from the 495/450 ratio.

We have found that the stable pH indicated by the fluorescence signals was independent of the amount of FD that the cells were permitted to endocytize. The mean (±SD) of 38 measurements was 4.75 ± 0.06.

Perturbation of intralysosomal pH

Fig. 6 shows the effects on intralysosomal pH of a 20-min perfusion with ammonia at three different concentrations followed by return to control medium. In each case there was a rapid increase in pH to a higher, relatively stable, value. The brief lag before the higher pH was attained is probably a reflection of the time necessary to exchange the perfusion fluid in the cell. The higher the ammonia concentration, the larger the pH increase. When the ammonia was removed from the perfusion fluid the pH dropped rapidly and actually attained a value slightly lower than that before the ammonia was added. During the next several minutes, the pH then rose to its initial value. This is reminiscent of the results shown in Fig. 5, where removal of amino acids (undoubtedly containing ammonia from glutamine breakdown) caused a transient drop in pH.

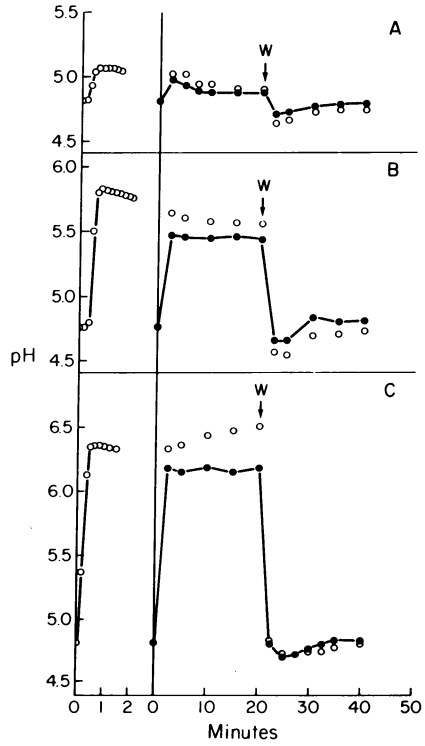

FIG. 6. Effect of ammonia on intralysosomal pH. Coverslips bearing macrophages containing FD in their lysosomes were removed from complete medium plus serum, placed in the perfusion chamber, and washed for 20 min in wash solution. They were then (time 0) perfused for 20 min with the same medium containing NH_4Cl at the following concentrations: (*A*) 100 μM; (*B*) 1 mM; (*C*) 10 mM. At the end of 20 min (arrow) the cells were perfused with wash solution. Open symbols, pH calculated by excitation at 495 nm; solid symbols, pH calculated from the 495/450 ratio (open symbols are omitted when they coincide with closed symbols).

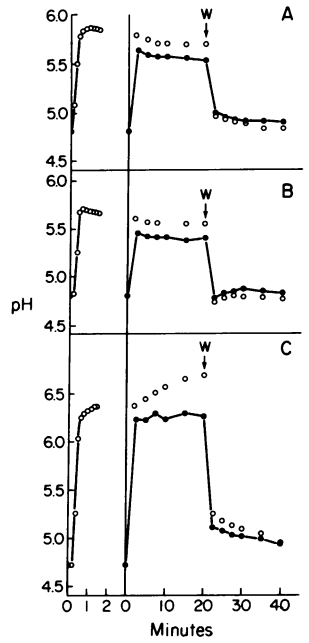

FIG. 7. Effect of weak bases on intralysosomal pH. Procedures are as in Fig. 6. (*A*) 1 mM tributylamine; (*B*) 500 μM amantadine; (*C*) 10 mM methylamine.

Fig. 7 shows the results of similar experiments with three

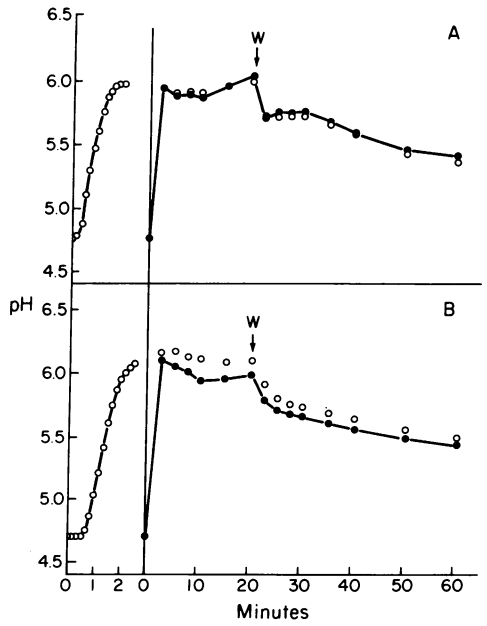

FIG. 8. Effect of acidic ionophores on intralysosomal pH. Procedures as in Fig. 6. (*A*) X537A at 5 μg/ml; (*B*) nigericin at 2 μg/ml.

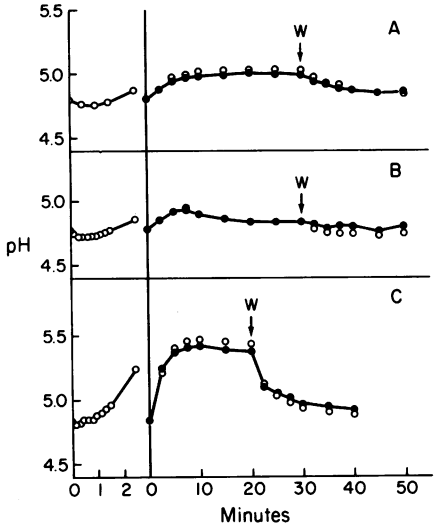

FIG. 9. Effect of metabolic inhibitors on intralysosomal pH. Procedures as in Fig. 6. (*A*) 50 mM 2-deoxyglucose; (*B*) 10 mM NaN_3; (*C*) 50 mM 2-deoxyglucose plus 10 mM NaN_3.

other weak bases. As was the case with ammonia, each of these weak bases caused a rapid increase in intralysosomal pH to a relatively stable value; when the base was removed from the perfusion fluid, the pH dropped rapidly back to a value close to its initial value. Both methylamine and amantadine cause vacuolation at the concentrations used here. In contrast, tributylamine does not cause vacuolation but rather inhibits the vacuolation caused by other compounds. We will return to this distinction in the discussion.

Fig. 8 shows the effects on intralysosomal pH of a 20-min exposure to the acidic ionophores X537A and nigericin. Both caused a substantial increase in pH. However, the response to these ionophores differed from that to the weak bases in two ways. The onset of the increase in pH was somewhat slower, and the reversal after the removal of the ionophores from the perfusion medium was very slow.

Fig. 9 shows the effects on intralysosomal pH of two metabolic poisons, alone and in combination. Neither azide nor 2-deoxyglucose had a very large effect on pH by themselves. In combination they caused an increase in pH that was considerably greater than the sum of their individual effects. The onset of the pH increase caused by these metabolic poisons was slower than any reported above, and the reversal, after the removal of the metabolic poisons from the perfusion fluid, appeared to be complete. Similar effects of these inhibitors were found even when the pH of the perfusion medium was as low as 6.6. This makes it likely that we are observing effects on the lysosomes themselves and not effects on any possible plasma membrane system for pH homeostasis in the cytoplasm. Similar effects were observed when cyanide was used in place of azide.

In all the experiments reported above, the excitation spectra measured on the FD in the lysosomes was recognizable in shape as belonging to the family of spectra in Fig. 2 or some intermediate thereof. Such was not the case when cells were exposed to chloroquine. As shown in Fig. 10, the 495-nm peak of the FD was shifted to longer wavelengths. This could not be explained by either light absorption or fluorescence of chloroquine in this spectral region. Similar shifts and quenching in the FD spectra were observed when the FD was dissolved in solutions containing chloroquine at 10 mM or more, but to some extent the magnitude of the spectral shifts depended on the ionic composition. By curve fitting, we estimated the pH inside the lysosomes when cells are exposed to chloroquine. Results of this experiment are shown in Fig. 11. Chloroquine caused a rapid, substantial increase in the intralysosomal pH that was only partially reversed when the chloroquine was removed from the perfusion fluid. Our interpretation of the spectra indicates that the concentration of chloroquine in the lysosomes was 50–100 mM all the time that the macrophages were exposed to chloroquine in the medium.

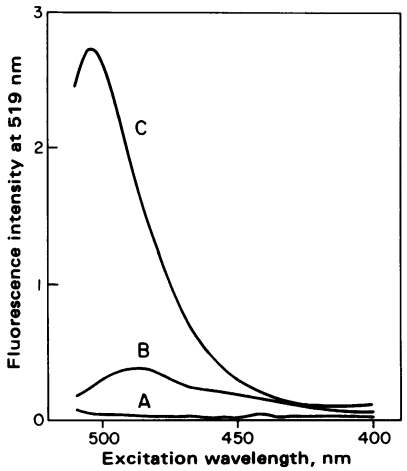

FIG. 10. Excitation spectra of macrophages containing FD in their lysosomes in the presence and absence of 100 μM chloroquine. Curve A, control cells; curve B, cells containing FD; curve C, cells containing FD exposed to 100 μM chloroquine for 10 min.

Kinetics of uptake of substances into lysosomes

Fig. 12 shows results of an experiment in which macrophages were exposed to two different compounds in the medium, 100 μM chloroquine and 10 mM methylamine. Under these conditions, each of these compounds causes clearly visible vacuolation in the macrophages within the time scale of this experiment. The kinetics of uptake were somewhat different in the two cases, as was the ratio of cellular to medium concentrations attained. Chloroquine is taken up most rapidly and reaches a plateau after 30 min. Methylamine is taken up more slowly and is only approaching a plateau after 2 hr. The ratio of concentration in cells to that in the medium was higher for chloroquine than it was for methylamine.

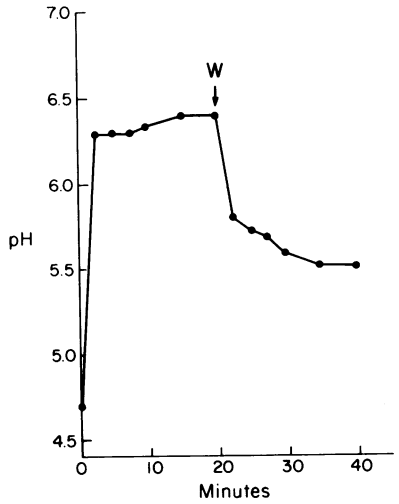

FIG. 11. Effect of chloroquine on intralysosomal pH. Procedures as in Fig. 6. The perfusion medium contained 100 μM chloroquine for the first 20 min.

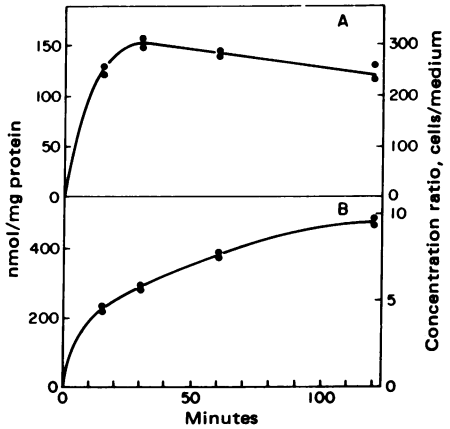

FIG. 12. Kinetics of uptake of various substances into macrophages. The concentration ratio, cell/medium, was calculated by assuming 5 μl of cell water for each mg of cell protein. (*A*) 100 μM chloroquine; (*B*) 10 mM methylamine.

DISCUSSION

In our calibration standards of FD, we cannot, of course, simulate exactly the chemical environment inside lysosomes. However, the insensitivity of FD fluorescence to environment that we have observed makes it likely that our values derived from the 495/450 ratio are accurate to within 0.1–0.2 pH unit. It remains highly desirable that measurements of this sort be repeated using a different fluorescence probe. If there is any heterogeneity in the pH of lysosomes, our values would be biased. Because the neutral excitation spectrum is so much more intense than the acidic one (Fig. 2), any FD in a very acidic compartment would be essentially invisible. Thus, our measured pHs are upper limits for the average.

The normal pH inside macrophage lysosomes seems to be 4.7–4.8. The transient lower pH seen during the recovery from ammonia treatment and after removal of amino acids (contaminated with ammonia from glutamine decomposition) from the medium may be a consequence of the intralysosomal production of protons as free base diffuses rapidly out through the lysosomal and plasma membranes and the protonated base inside dissociates.

The theory of uptake of weak bases by lysosomes (7) predicts that back diffusion of protonated base will limit the concentration ratio that can be achieved and, at the same time, provide a proton leak. Indeed, we find that methylamine and ammonia, both substances that are concentrated very weakly in lysosomes, cause a great increase in the intralysosomal pH. Amantadine, a substance that is concentrated much more strongly, causes a substantially smaller increase in pH at comparable intralysosomal concentrations. It seems likely that the inability of tributylamine to cause vacuolation, its ability to inhibit the vacuolation caused by other weak bases, and its ability to increase the intralysosomal pH all are consequences of the lipophilic nature of its protonated form. This permits rapid proton leakage in the presence of this compound.

The large pH increase in lysosomes after exposure to the acidic ionophores nigericin and X537A makes it likely that the cellular vacuolation caused by these compounds is a consequence of the exchange of potassium for protons across the lysosomal membrane. In this way, an active process of proton transport into lysosomes could be coupled to potassium transport in the same direction and lead to osmotic swelling.

The pH values measured in the presence of chloroquine are much less precise than the others we have reported. There is, however, no doubt that this drug causes a substantial increase in the intralysosomal pH. Our pH value is remarkably close to the value reported by Reijngoud and Tager (15) for isolated lysosomes exposed to chloroquine at the same concentration. However, this coincidence is almost certainly fortuitous because the lysosomes of the living cells started with a lower internal pH and took up about 10 times more chloroquine than did the isolated lysosomes (8, 15).

The results reported here lend support to the hypothesis that the low intralysosomal pH is maintained, at least partially, by an active process. First, the normal pH of 4.7–4.8 that we find in lysosomes is considerably lower than that found in isolated lysosomes (2–4). More direct evidence comes from the effects of azide or cyanide and 2-deoxyglucose alone and together. Neither type of inhibitor seems to have much effect alone, but together they act synergistically to increase the intralysosomal pH. This effect is most easily explained as the consequence of depriving the cells of both sources of ATP, oxidative phosphorylation and glycolysis. Another argument for an active process of proton transport comes from a comparison of kinetics of pH change (Fig. 7) and bulk uptake of methylamine (Fig. 12). The pH changes, presumably a consequence of simple acid-base equilibria of the same class as the Donnan equilibrium, are very rapid. Uptake of the base is very much slower and continues while the pH in the lysosomes remains relatively constant. This is most easily explained by an active pumping of protons into the lysosomes, thus permitting further uptake of methylamine even though the pH remains constant. The pH increase seen in the lysosomes of cells exposed to weak bases is probably the consequence of proton leakage as a consequence of permeation by protonated base.

We are indebted to Mihoko Ohkuma for expert technical assistance; to Shigeru Sassa for useful suggestions; to Attallah Kappas for the use of his spectrofluorometer; to Edward Reich for the use of his thermostated cell holder; and to Christian de Duve for advice and encouragement. This work was supported by Grants AG 00367 and AG 00609 from the U.S. Public Health Service and by Grant PCM76-16657 from the National Science Foundation.

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