

Effect of Weak Bases on the Intralysosomal pH in Mouse Peritoneal Macrophages

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ABSTRACT The spectral characteristics of dextran, labeled with fluorescein, depend upon pH. We have loaded the lysosomes of mouse peritoneal macrophages with this fluorescence probe and used it to measure the intralysosomal pH under various conditions. The pH of the medium has no effect on the intralysosomal pH. Weakly basic substances in the medium cause a concentration-dependent increase in the intralysosomal pH. However, the concentration of base necessary to produce a significant change in the intralysosomal pH varies over a wide range for different bases. The active form of the base is the neutral, unprotonated form. Although most of these weak bases cause an increase in the volume of the lysosomes, increase in lysosomal volume itself causes only a minor perturbation of the intralysosomal pH. This was demonstrated in cells whose lysosomes were loaded with sucrose, and in cells vacuolated as a consequence of exposure to concanavalin A.

The results of these studies are interpreted in terms of energy-dependent lysosomal acidification and leakage of protons out of the lysosomes in the form of protonated weak bases.

In a previous paper (6) we reported the results of experiments showing that exposure of macrophages to medium containing any one of a large number of weak bases results in the uptake of the bases into lysosomes, and, when the concentration in lysosomes becomes sufficiently high, osmotic swelling of these particles to form large phase-lucent vacuoles occurs. We showed that the concentration of the free base in the medium is the determining factor for uptake and vacuolation, and that the protonated base in the medium has no effect. Widely different concentrations are required to elicit vacuolation by different bases.

We have described previously a fluorescence probe technique for the measurement of intralysosomal pH (5). In this study we use this technique to explore, in some detail, the pH changes caused in the lysosomes by some of these weak bases, and we discuss the results in terms of the leakage of protons out of lysosomes in the form of protonated base. We conclude that the high acidity in lysosomes is the consequence of an active process of proton secretion into lysosomes.

MATERIALS AND METHODS

Cell Culture

Our culture procedures for mouse peritoneal macrophages, adapted from

This paper is appearing without final revision due to the untimely death of Dr. Brian Poole.

Cohn and Benson (2), have been described fully in the previous paper in this series (6).

Lysosomal pH Measurement

This technique has been described in more detail elsewhere (5). Briefly, cells were exposed to medium containing 1 mg/ml fluorescein-labeled dextran for 24 h. They were then washed free of medium and the cover slips were mounted in a special holder in a regular fluorescence cell that was perfused with various media. The fluorescence of the cells was measured with a Hitachi Perkin-Elmer MPF4 or MPF44A in ratio mode. Fluorescence emission was always measured at 519 nm. The ratio of fluorescence observed when excitation was at 495 nm to that observed when excitation was at 450 nm was used to determine intralysosomal pH (except for the experiment represented in Fig. 6, where the fluorescence intensity at 495 nm was also used).

Within the lysosomes of cells exposed to weak bases, the local concentration of the base is very high. Consequently, it was necessary to verify, for each base studied, that these high concentrations did not, in any way, affect our fluorescence measurements. We found that the weak bases atropine, eserine, and propranolol interfered with the fluorescence measurements, and we will present no data on their effects on intralysosomal pH. These bases showed interesting properties when their uptake and their ability to produce vacuoles was studied (6). Chloroquine interfered to some extent with the fluorescence measurements, but we were able to correct for this effect as described previously (5).

RESULTS

Fig. 1 shows the results of an experiment in which the pH inside lysosomes was measured in cells exposed to media of various pHs. Medium pH did not have any obvious effect on the intralysosomal pH.

Fig. 2 shows the changes in intralysosomal pH caused by exposure of the cells to medium containing 10 mM ammonium chloride, followed by return to control medium. The pH increased rapidly as soon as the ammonium chloride was applied, remained constant during 2 h of exposure, and then returned rapidly to normal as the ammonium chloride was washed out. Thus there is a stable intralysosomal pH associated with exposure of the cells to a particular concentration of a weak base.

We have measured the intralysosomal pH in cells exposed to a wide range of concentration of a number of weak bases. The results of these experiments are shown in Figs. 3 and 4. In Fig. 3 we have plotted, on a logarithmic scale, the measured intralysosomal pH against the total concentration of bases in the medium. Chloroquine caused comparable increases in lysosomal pH at much lower concentrations than did tributylamine, ammonium chloride, and methylamine. When the pH of the medium was raised, the tributylamine and methylamine caused a larger increase in lysosomal pH. In Fig. 4 we have plotted the same results as well as results for benzylamine, ethylamine, and NH_4Cl . In this figure we have plotted the intralysosomal pH against the concentration of free, unprotonated base. We see now that for tributylamine and methylamine the results obtained at different values of the medium pH fall on the same line. Thus, for any particular compound it is the concentration of free base that determines the intralysosomal pH and not the total concentration of the base. However, for different compounds, the concentration of free base required

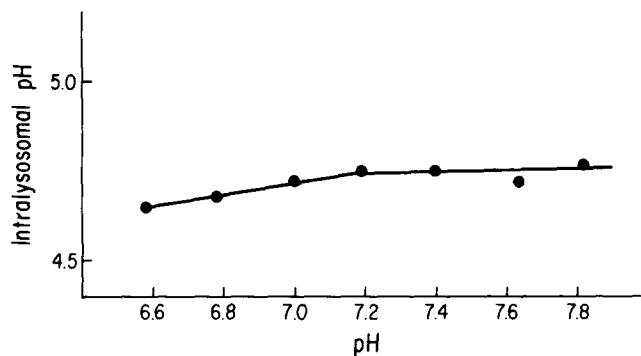


FIGURE 1 The effect of the pH of the medium on the intralysosomal pH. Measurements were made 20 min after the cells had been exposed to medium of the indicated pH.

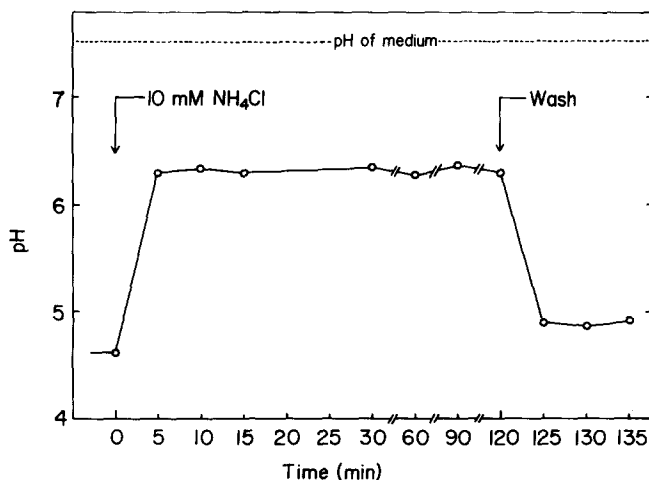


FIGURE 2 The effect of 10 mM NH_4Cl on intralysosomal pH. After 2 h of exposure (arrows) the cells were returned to control medium.

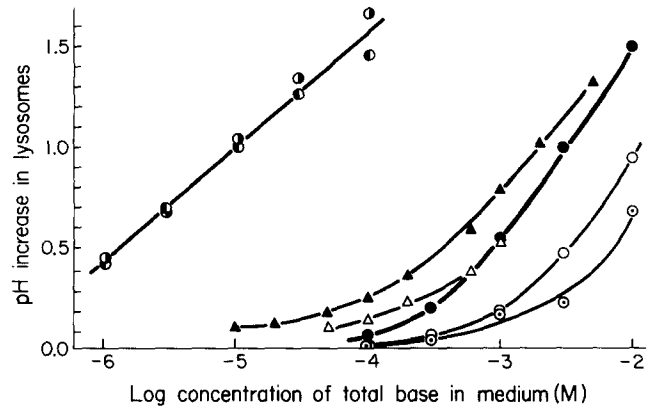


FIGURE 3 The effect of the total medium concentration of a number of different amines on the intralysosomal pH. The pH was measured after 10 min exposure to the compounds in medium of the indicated pH. Methylamine: (●) pH 7.6; (○) pH 7.0; (◐) pH 6.6. Tributylamine: (▲) pH 7.6; (△) pH 7.0. Chloroquine, pH 7.6 (calculated from calibration standards prepared with two salt forms of chloroquine): (◐) diphosphate; (●) dichloride.

to produce the same pH increase in the lysosomes varies over a wide range. The concentration of chloroquine free base required to cause an increase in intralysosomal pH was much lower than that for the other weak bases.

Fig. 5 shows the results of an experiment in which cells were exposed to medium containing concanavalin A or sucrose, and the intralysosomal pH was measured at various times. These are both compounds that cause a large increase in the volume of the lysosomal system, but they are not weak bases. Both of these compounds caused a small increase in intralysosomal pH, but these increases took a long time to occur. The kinetics were completely different from those shown in Fig. 2. A similar lack of substantial, rapid perturbation of the intralysosomal pH was observed when the cells were exposed to 10 mM Tris, aniline, tetraethylammonium chloride, or 20 μM valinomycin.

Fig. 6 shows the results of an experiment in which cells were exposed to 200 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) for 20 min. The pH in the lysosomes rose over a period of 2 min. After the cells were returned to control medium, the pH dropped slowly, but even after 40 min it still had not returned to the initial value.

In one experiment, we investigated the effect of temperature on intralysosomal pH. Two cover slips (initial lysosomal pH 4.28 and 4.83) were kept on ice for 1 h. The intralysosomal pH was then measured to be 5.45 for both cover slips. (Of course, fluorescence calibration curves were determined for the reduced temperature.) Sluggish response of the thermostatic equipment available precluded studies of the kinetics and reversibility of this effect.

DISCUSSION

It would appear from the results in Fig. 1 that whatever the mechanism of maintenance of low pH inside lysosomes is, it is affected only slightly by the pH of the medium. It has been observed before, in ascites tumor cells (7) and in muscle (1, 11), that cytoplasmic pH is not strongly affected by medium pH. The fortunate consequence of this result is that we can manipulate the relative concentration of free and protonated base in the medium by manipulating medium pH without producing much direct effect on the intralysosomal pH.

We have shown previously (5) that when cells are exposed

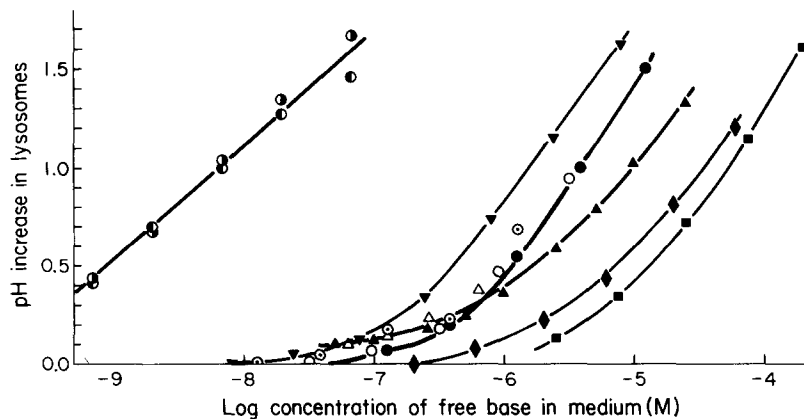


FIGURE 4 The effect of the medium concentration of the free base forms of various amines on intralysosomal pH. The pH was measured after 10 min exposure of cells to the compounds in medium of the indicated pH. Methylamine: (●) pH 7.6; (○) pH 7.0; (⊙) pH 6.6. Tributylamine: (▲) pH 7.6; (△) pH 7.0. NH_4Cl : (■) pH 7.6. Triethylamine: (▼) pH 7.6. Benzylamine: (◆) pH 7.6. Chloroquine, pH 7.6 (calculated from calibration standards prepared with two salt forms of chloroquine): (◐) diphosphate; (◑) dichloride.

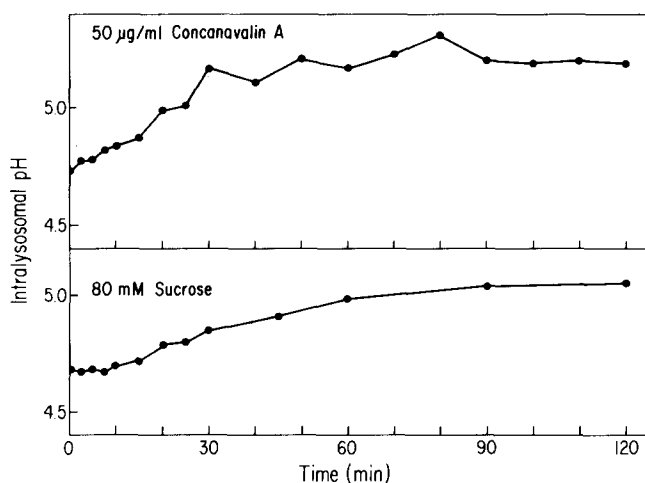


FIGURE 5 The effects of concanavalin A and sucrose on intralysosomal pH.

to a variety of weakly basic substances in the medium, the pH within the lysosomes rises rapidly to a new plateau value stable for 20 min. In Fig. 2 we show that the elevated pH value can remain stable for as long as 2 h and that the effect is still rapidly reversible. This means that we can associate, meaningfully, a particular intralysosomal pH with a particular concentration of weak base in the medium.

In Fig. 3 we have plotted the pH increases that we measured against the total concentrations of three weak bases. Chloroquine caused a measurable pH increase even at $1 \mu\text{M}$ concentration. Considerably higher concentrations of methylamine and tributylamine were required to produce the same effect. For both methylamine and tributylamine, larger pH shifts were observed at higher medium pH when the total base concentration remained the same.

In Fig. 4 we have plotted these same data, as well as data for three other amines, against the concentration of free base in the medium. Here we see that all the data for methylamine and tributylamine fall on the same lines. This shows that it is the free base that is responsible for the pH shift and that the protonated base has no effect. It is also clear from Fig. 4 that very different concentrations of free base are required of different bases to produce the same effect. This dependence was observed previously for vacuolation and base uptake (6).

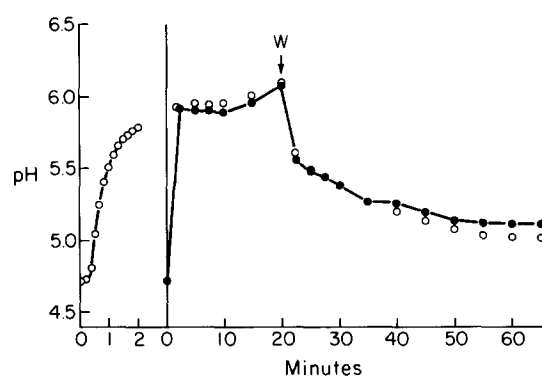


FIGURE 6 The effect of 200 M CCCP on the intralysosomal pH. (●) pH calculated from fluorescence ratio; (○) pH calculated from fluorescence intensity. (For details see reference 5.)

The mechanism of pH change, base uptake, and vacuolation of lysosomes can be explained most simply by the model illustrated in Fig. 7 (3, 4). In the absence of base, some energy dependent mechanism transports protons into lysosomes producing a gradient of proton concentration across the membrane. In the presence of weak base, the free base diffuses rapidly across the plasma and lysosomal membranes. Because of the acidity inside the lysosomes, base is trapped there by protonation and the intralysosomal concentration builds up. (The lysosomal membrane is much less permeable to the protonated form than it is to the free base.) Osmotic influx of water causes the lysosomes to swell. However, finite diffusion rates of the protonated form out of the lysosome down the very large concentration gradient that is formed, limit the process and increase the intralysosomal pH by transporting protons out, thereby partially short-circuiting the "proton pump." Differences in back diffusion rates of different bases explain the different response of pH change (Fig. 4), vacuolation, and base uptake (6).

We have shown in previous papers that the uptake of weak bases into lysosomes is a progressive process taking from 20–60 min or longer to go to completion (6, 12). This is in contrast to the rapid transition to a new stable intralysosomal pH shown in Fig. 2 for ammonia and in a previous paper for a number of other weak bases (5). In Fig. 8 we have plotted data taken from our earlier work (5, 6) showing changes with time in the intralysosomal pH and in the cellular content of methylamine

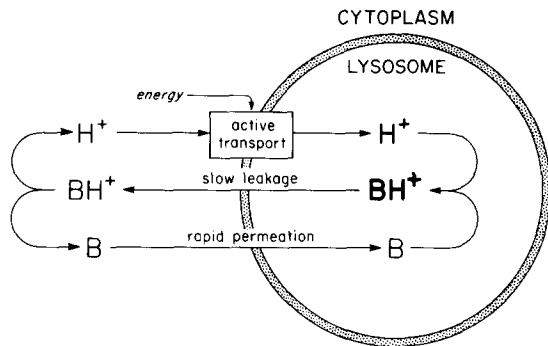


FIGURE 7 Schematic model of lysosomal pH maintenance and the uptake of weak bases.

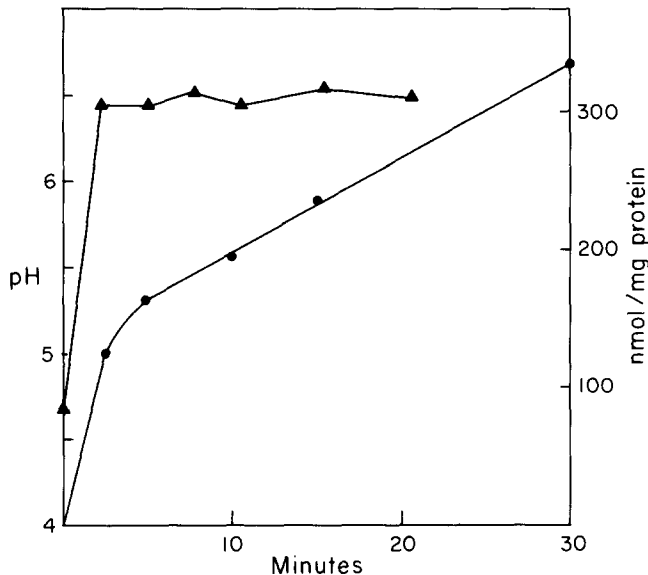


FIGURE 8 Changes with time of methylamine content and intralysosomal pH in cells exposed to 10 mM methylamine. (●) methylamine content; (▲) intralysosomal pH. Data from references 5 and 6.

in cells exposed to 10 mM methylamine. The lysosomal pH remained constant during the time when more and more base was accumulating inside lysosomes. Thus the pH changes and uptake of bases we observe in the lysosomes of living cells cannot be a simple consequence of the neutralization by the weak base of lysosomal buffering capacity as suggested by Reijngoud and Tager (8) on the basis of their *in vitro* studies with chloroquine and methylamine.

It seems that there are two phases to the uptake of weak bases into lysosomes. Rapid attainment of a steady state occurs where protonated base leakage balances proton pumping with the pH inside the lysosomes at a steady, elevated value. Then base accumulation continues and the lysosomal compartment swells progressively to provide room for more base. These processes involve membrane reorganization; either the fusion of many small lysosomes to form larger vacuoles with greatly reduced surface-to-volume ratio, or recruitment of membrane from some other source.

The nature of the active process whereby protons are accumulated in lysosomes is unclear. That the process is energy dependent is suggested by our previous finding of increased intralysosomal pH in the presence of metabolic poisons (5) and the effect of low temperature reported here. Moreover, isolated lysosomes from rat liver can maintain only ~1 pH unit differ-

ential across the membrane (8) whereas here we have a differential of at least 2.3. An ATPase activity has been reported in isolated lysosomes from rat liver (9), and it has been reported that ATP will increase methylamine uptake in isolated lysosomes (10). However, the salt requirements for the two effects were not the same. There is no reason why ATP should necessarily be the source of energy for proton accumulation, but the existence of some active mechanism for proton accumulation seems indisputable.

If the leakage rate of protons out of lysosomes is low, the pH within the lysosomes could be maintained with little expenditure of energy in normal cells. The situation is, however, very different in the presence of weak bases. It is interesting to estimate how much cellular energy is necessary to replace the protons that are consumed in the protonation of the incoming free base. From results reported in the previous paper in this series (6), we can estimate that the initial uptake of methylamine into cells is at least 50 nmol/min per mg protein, when the concentration of this compound in the medium is 10 mM. From the data of Figs. 3 and 4 we can see that the pH difference under these conditions is ~1.5. Therefore, the difference between the chemical potentials of protons inside and outside lysosomes (ignoring possible effects of a membrane potential) is:

$$\Delta\mu = RT \ln (H_i/H_o) = 2(310) \ln 32 = 2.15 \times 10^3 \text{ cal/mol.}$$

Thus the work performed is:

$$5 \times 10^{-8} (2.15 \times 10^3) = 1.07 \times 10^{-4} \text{ cal/min/mg protein.}$$

Loike and Silverstein¹ have measured the heat production of mouse peritoneal macrophages. They found a value of ~1 mcal/min per mg protein. Thus, even under the stress of exposure to 10 mM methylamine, the additional work of pumping protons into lysosomes seems well within the capacity of the cells.

Although an increase in the intralysosomal pH is often accompanied by vacuolation, this is not always the case. For example, tributylamine causes no vacuolation, but rather, inhibits vacuolation and uptake caused by other amines (6). Presumably, the back diffusion of the protonated form of this compound is so rapid that accumulation does not occur. On the other hand, mere vacuolation, as shown in Fig. 5 for sucrose or concanavalin A, does not cause any rapid change in pH.

As shown in Fig. 6, the protonophore CCCP also increases the intralysosomal pH, presumably by transporting protons out of the lysosomes. Reijngoud and Tager (8) found little effect of this compound on the pH inside isolated lysosomes, but this is not surprising because the membrane of these lysosomes seems to be freely permeable to protons.

Some of the compounds studied here have important biological effects. We will defer a discussion of the relevance of changes in the intralysosomal pH to these effects until we have examined, in a subsequent paper, their effects on lysosomal protein degradation.

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¹ Loike, J., and S. Silverstein. Personal Communication.

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