# Weak Bases and Ionophores Rapidly and Reversibly Raise the pH of Endocytic Vesicles in Cultured Mouse Fibroblasts

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ABSTRACT    It has been shown that endocytic vesicles in BALB/c 3T3 cells have a pH of 5.0 (Tycko and Maxfield, *Cell*, 28:643–651). In this paper, a method for measuring the effect of various agents, including weak bases and ionophores, on the pH of endocytic vesicles is presented. The method is based on the increase in fluorescein fluorescence with 490-nm excitation as the pH is raised above 5.0. Intensities of cells were measured using a microscope spectrofluorometer after internalization of fluorescein-labeled $\alpha_2$-macroglobulin by receptor-mediated endocytosis. The increase in endocytic vesicle pH was determined from the increase in fluorescence after addition of various concentrations of the test agents. The following agents increased endocytic vesicle pH above 6.0 at the indicated concentrations: monensin (6 $\mu$M), FCCP (10 $\mu$M), chloroquine (140 $\mu$M), ammonia (5 mM), methylamine (10 mM). The ability of many of these agents to raise endocytic vesicle pH may account for many of their effects on receptor-mediated endocytosis. Dansylcadaverine caused no effect on vesicle pH at 1 mM. The observed increases in vesicle pH were rapid (1–2 min) and could be reversed by removal of the perturbant. This reversibility indicates that the vesicles themselves contain a mechanism for acidification. The increase in vesicle pH due to these treatments can be observed visually using an SIT video camera. Using this method, it is shown that endocytic vesicles become acidic at very early times (i.e., within 5–7 min of continuous uptake at 37°C).

A wide variety of ligands, including certain hormones, serum proteins, bacterial toxins, lysosomal enzymes, and enveloped viruses, enter cells via receptor-mediated endocytosis (reviewed in references 1 and 2). For many of these ligands, the pathway of entry into the cell has been shown to begin with the concentration of occupied receptors over clathrin-coated pits, followed by entry into endocytic vesicles (1, 2). In several cases, there is evidence that receptors enter endocytic vesicles along with the ligand (3–5).

Several workers have examined the effect of weak bases or ionophores on various steps in the receptor-mediated endocytosis pathway. A variety of effects have been reported, including reduced rates of endocytosis (6, 7), inhibition of receptor recycling (8–11), recycling of intact ligand-receptor complexes to the surface (4), inhibition of diphtheria toxin toxicity (12), and reduction of virus infectivity (13).

Since acidification is an early event in the endocytosis pathway (16), it is plausible that weak bases and ionophores could exert some of their effects by raising endocytic vesicle pH. To explore that possibility, I have directly measured the ability of several of these agents to raise endocytic vesicle pH. In general, the concentrations required to raise vesicle pH are similar to concentrations where various effects on the endocytosis pathway have been reported. The effects on vesicle pH are rapidly

reversible. A notable exception is dansylcadaverine which does not raise vesicle (or lysosomal) pH at concentrations far above those which inhibit endocytosis (7), recycling (10), or viral infectivity (13).

The results presented in this paper indicate that vesicle acidification is a very early event after internalization and that endocytic vesicles contain a mechanism for regulating their internal pH. These results also indicate that acidification of endocytic vesicles may play a central role in the processing of many ligands and receptors.

## MATERIALS AND METHODS

### Materials

Monodansylcadaverine was obtained from Fluka, monensin from Calbiochem, and chloroquine diphosphate from Boehringer Mannheim. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), methylamine, propylamine, and hexylamine were obtained from Sigma Chemical Co., St. Louis, MO. $\alpha_2$-Macroglobulin ($\alpha_2$M) and fluorescein-labeled $\alpha_2$M (F-$\alpha_2$M) were prepared as described previously (16).

### Cell Culture

BALB/c 3T3 mouse fibroblasts were grown in Dulbecco-Vogt modified Eagle's medium (DME; Gibco, Grand Island Biological Co., Grand Island, NY) containing 10% (vol/vol) calf serum at 37°C in an atmosphere of 95% air and 5%

CO₂. Cells for binding studies were plated in 35 mm dishes (Falcon Labware, Oxnard, CA) or 24-well plates (Costar, Data Packaging, Cambridge, MA). Culture dishes for fluorescence microscopy were prepared by punching 7-mm holes in 35-mm tissue culture dishes and attaching coverslips to the bottom surface using a mixture of paraffin (Tissue-Tek, Fisher) and petroleum jelly (Vaseline) (3:1, vol:vol) to form a watertight seal (15). The dishes were sterilized by ultraviolet irradiation. Cells were plated at ~1 × 10⁵ cells/dish and were used 2–4 d after plating.

## Quantitative Fluorescence Microscopy

Fluorescence intensities were measured using a Leitz MPV Compact microscope spectrofluorometer mounted on a Diavert microscope. A 75 W xenon lamp was used for epifluorescent illumination. The microscope was modified by inserting a beam splitter to allow simultaneous intensity measurement and observation using an image intensifier television camera. A standard Leitz fluorescein filter cube was modified by removing the excitation filter and placing either a 490 or a 450-nm narrow bandpass filter in the incident light path. The incident beam was centered and stopped down to a diameter of ~50 μm, and the position of the beam was noted on the video monitor. The measuring diaphragm was adjusted to a diameter slightly smaller (in the image plane) than the illuminated area. pH measurements on endocytic vesicles and lysosomes were made as described previously (16) using the ratio of fluorescein fluorescence intensities with 450 and 490-nm excitation.

To determine the effect of various agents on endocytic vesicle pH, cells were incubated with F-α₂M (100 μg/ml) in DME for 14 min at 37°C. The cells were rinsed four times with warm DME and placed in 1 ml of measuring buffer (155 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 10 mM glucose, 20 mM HEPES, pH 7.4). The cells were immediately placed on the microscope stage and observed with a 63 power, N.A. 1.3 objective, and a single cell, or a group of 2–4 cells, was centered (under bright field illumination) in the measuring area.

An initial intensity measurement was made using 490-nm excitation and an illumination period of 0.1 s. Immediately after this measurement, 1 ml of measuring buffer containing the test agent was added to the dish. The medium was gently mixed with a Pasteur pipet, taking care not to move the dish. Intensity measurements were made 1, 2, and 3 min after addition of the test agent. After the third measurement, an additional 1 ml of measuring buffer was added which contained methylamine hydrochloride (50 mM final concentration). Intensity measurements were made 1, 2, and 3 min after adding the methylamine. The total time from the addition of F-α₂M to the final measurement did not exceed 22 min. The total fluorescence illumination time was 0.7 s. The video system was used to check for movement of the cells after the initial measurement and to check for F-α₂M binding to extracellular debris in the measurement field. Video images were recorded, displayed, and photographed as described previously (16).

## Binding Studies

α₂M was iodinated by the chloramine-T procedure (17) to a specific activity of 2 × 10⁵ cpm/μg. ¹²⁵I-α₂M was separated from Na ¹²⁵I by chromatography on a Sephadex G-25 column. Epidermal growth factor (EGF) was prepared by the method of Savage and Cohen (18). ¹²⁵I-EGF was prepared by the method of Carpenter and Cohen (19) with a sp act of 4.1 × 10⁴ cpm/ng. Measuring buffer was adjusted to a range of pH values from 6.4 to 7.8 using HCl or NaOH. Confluent monolayers were rinsed with ice-cold measuring buffer and incubated on ice with ¹²⁵I-α₂M (3 nM) or ¹²⁵I-EGF (2 nM) in measuring buffer with 1% bovine serum albumin. Incubations were continued on ice, with constant shaking, for 1 h (EGF) or 4 h (α₂M). At the end of the incubation, cells were rinsed four times with ice-cold measuring buffer and dissolved in 1 N NaOH. Radioactive counts were measured on a Beckman 4000 gamma counter (Beckman Instruments, Inc., Fullerton, CA). Nonspecific binding was determined by competition with excess α₂M (2 mg/ml) or EGF (2 μg/ml). All values were determined in quadruplicate.

## RESULTS

The pH-dependence of the excitation profile of fluorescein makes it a very useful probe for measuring intracellular pH (14). Fig. 1 shows the pH dependence of fluorescein labeled α₂M (F-α₂M) fluorescence using 450 or 490-nm excitation in the instrument used for these studies. Between pH values of 4.5 and 7.5, the fluorescence excited at 450 nm is relatively independent of pH, but the 490-nm fluorescence increases sharply with increasing pH. In a previous study (16), the ratio I₄₅₀/I₄₉₀ was used to estimate the pH of endocytic vesicles, and we obtained a value of 5.0 ± 0.2 (SE) after a 15-min continuous
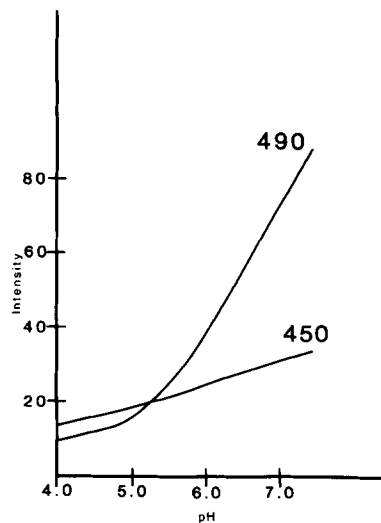


FIGURE 1 pH Dependence of F-α₂M fluorescence. Cells were incubated with F-α₂M (100 μg/ml) in DME for 30 min at 37°C, rinsed, and fixed in 2% formaldehyde. The cells were then equilibrated in buffers at pH 4, 5, 6, 7, and 7.5 in the presence of 10 mM methylamine. Fluorescence intensities were measured from 20 cells in each dish with 450 nm and 490 nm excitation.

TABLE I

Concentrations Required for Half-Maximal Increase in 490-nm Fluorescence

| Agent | $C_{0.5}^*$ |
|---|---|
| | $M$ |
| Monensin | $6 \times 10^{-6}$ |
| FCCP‡ | $1 \times 10^{-5}$ |
| Chloroquine | $1.4 \times 10^{-4}$ |
| Ammonia | $5 \times 10^{-3}$ |
| Propylamine, hexylamine, methylamine | $1 \times 10^{-2}$ |
| Dansylcadaverine | $>2 \times 10^{-3}$ |

* The concentrations listed gave $\Delta I$ values of 0.5. This corresponds to a rise in vesicle pH from ~5.0 to 6.2. Each value of $C_{0.5}$ was obtained from 3–5 concentration curves similar to those in Fig. 3. The range of values was approximately ±20% of the average values.

‡ Unlike the other agents listed, FCCP at higher concentrations did not give $\Delta I$ values near 1 (see Fig. 3). The range of maximum values was 0.5–0.8 (five experiments).

uptake and 5-min chase with F-α₂M. In that study, light and electron microscopy were used to demonstrate that α₂M is in nonlysosomal structures at the time of measurement.

To measure the effect of various agents on endocytic vesicle pH, cells were incubated with F-α₂M for 14 min, and then the effect of the test agent on the intensity of fluorescein emission with 490-nm excitation was measured (see Materials and Methods). Agents which raise endocytic vesicle pH should increase the fluorescence intensity, with the extent of the increase dependent on the change in pH. We measured the fluorescence intensity (0.1-s illumination) on cells before and after the addition of various agents (listed in Table I). Within 1–2 min after addition, the fluorescence reached a stable value. We then added a high concentration of methylamine (50 mM) to the cells to elicit a 'maximal' increase in the vesicle pH and again measured the intensity. The increase in intensity caused by the test agent was then compared to the increase caused in the same cells by the high concentration of methylamine. The relative increase, $\Delta I$, was defined as:

$$\Delta I = (I_{Test} - I_{Init})/(I_{Max} - I_{Init})$$

where $I_{Test}$ is the stable intensity value after addition of the test agent, $I_{Init}$ is the intensity before addition of the agent, and $I_{Max}$ is the intensity after addition of 50 mM methylamine.

This procedure was chosen for several reasons. A microscope spectrofluorometer was used because it allows one to observe

the cells as they are measured. Because of the pH dependence of fluorescein fluorescence, even a small amount of F-α2M bound extracellularly to debris or dead cells would make a large contribution to the signal compared with F-α2M at pH 5. Any measurement based on small numbers of cells must be internally calibrated to avoid artifacts arising from cell-to-cell variability in α2M uptake (20). In our previous study, internal calibration was provided by using the ratio $I_{450}/I_{490}$ which is independent of total uptake. In the present study, internal calibration was provided by comparing the effect of the test agent to the effect of 50 mM methylamine on the same cells. The value of ΔI should be independent of total F-α2M uptake. The average value of $I_{Max}/I_{Init}$ was 1.7. Addition of the agents listed in Table I to cells which had not been incubated with F-α2M caused no change (±3%) in the cellular autofluorescence.



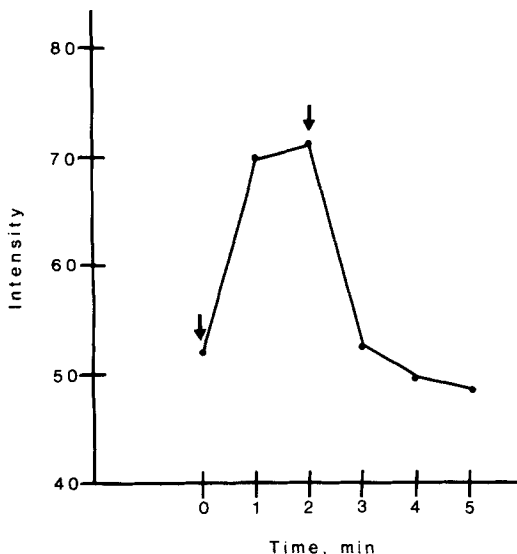FIGURE 2  Time dependence and reversibility of monensin effects on endocytic vesicle pH. Cells were incubated with F-α2M (100 μg/ml) for 14 min at 37°C, rinsed with measuring buffer, and placed on the microscope photometer. The intensity of a single cell was measured with 490 nm excitation, and monensin (10 μM final concentration) was added to the medium. The fluorescence intensity from the same cell was measured 1 and 2 min after addition of monensin. The cells were immediately rinsed three times with measuring buffer without monensin, and two subsequent measurements were made from the same cell. The time scale in the figure starts with the addition of monensin.
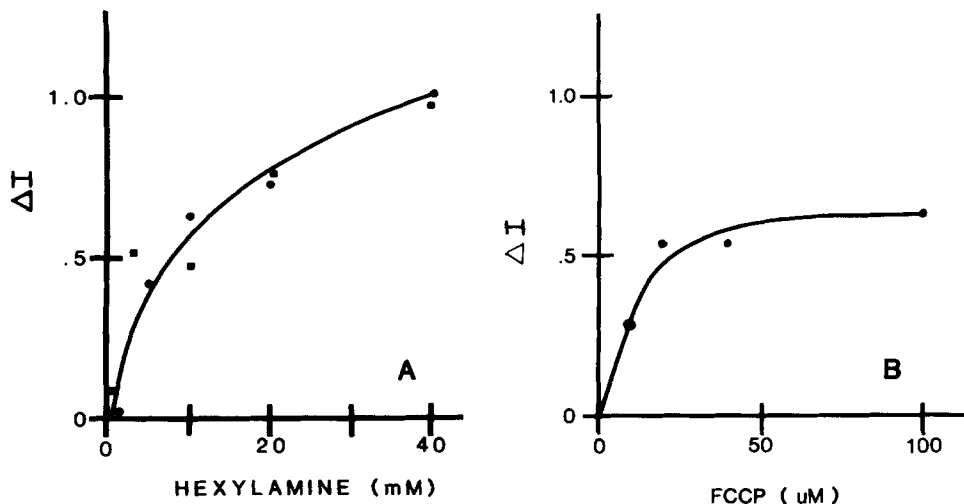
Also, these agents do not, by themselves, change the fluorescence of F-α2M. An advantage of the present method is that cellular autofluorescence, scattered light, and extracellular F-α2M should all contribute equally in each intensity measurement. Therefore, these sources all cancel out in the evaluation of ΔI. A disadvantage is that pH values cannot be determined directly from ΔI. However, by using Fig. 1 and measuring the pH in the presence or absence of 50 mM methylamine, one can estimate the pH from ΔI. As previously described, the pH in endocytic vesicles is 5.0 (16). Using the same method (i.e., measuring $I_{450}/I_{490}$), a pH of 7.0 ± 0.2 was obtained in the presence of 50 mM methylamine.

Ohkuma and Poole have measured intralysosomal pH in macrophages by placing a monolayer of cells in a modified fluorescence cuvette (14, 21). When measuring stable pH values, they used the shape of the fluorescein excitation profile, but for rapid time-dependent processes they measured changes in $I_{495}$. They reported essentially equivalent results with the two methods. They proposed that weak bases, such as chloroquine or ammonia, raise lysosomal pH by diffusing across membranes as the uncharged species and binding protons within the acidic organelles.

## Time and Concentration Dependence

Fig. 2 shows the effect of 10 μM monensin on the intensity of the fluorescence from F-α2M in endocytic vesicles. The intensity rapidly increases to a new stable value, indicating a rise in the intravesicle pH. Removal of monensin from the medium by extensive rinsing returns the fluorescence intensity to approximately the initial value. For all of the agents tested, stable intensity values were obtained within 1–2 min after addition. The increase in fluorescence caused by addition of amines was also reversible (not shown). The rapid rise in pH and the rapid reversibility are in general agreement with results obtained in measurements of changes in lysosomal pH (14, 21).

The concentration dependence of ΔI for hexylamine and the proton ionophore FCCP are shown in Fig. 3. Curves similar to that shown for hexylamine were obtained for all of the agents listed in Table I except FCCP and dansylcadaverine, and the concentrations which gave ΔI = 0.5 are listed. The electrogenic proton ionophore, FCCP, produced only a partial increase in ΔI even at high concentrations (Fig. 3 B). Monensin, an electroneutral ionophore, gave ΔI values near 1.0 at concentrations >15 μM. For monodansylcadaverine, I found ΔI = 0 for concentrations as high as 1 mM. This concentration is substan-



FIGURE 3  Concentration dependence of hexylamine and FCCP effects on endocytic vesicle pH. Cells were incubated with F-α2M, rinsed, and placed on the microscope photometer as described in Fig. 2. After the first intensity measurement, various concentrations of hexylamine (A) or FCCP (B) were added to the measuring buffer, and three intensity measurements were made at 1-min intervals. After the third measurement, 50 mM methylamine was added to the measuring buffer, and three more intensity measurements were made. ΔI, the relative increase in fluorescence with 490 nm excitation, was calculated as described in text.

tially higher than the concentrations required to affect the behavior of several ligand-receptor systems (1, 7, 10, 13). A substantial rise in fluorescence excited at 490 nm was observed when 50 mM methylamine was added to cells in the presence of 1 mM monodansylcadaverine, indicating that dansyl fluorescence would not interfere with the detection of $\Delta I$. Monodansylcadaverine (500 $\mu$M) also failed to raise lysosomal pH significantly in human fibroblasts (22) or in BALB/c 3T3 cells (not shown).

In the presence of 50 mM methylamine, the pH of endocytic vesicles was $7.0 \pm 0.2$ (3 experiments, 18 cells). If one takes this as the pH value when $\Delta I = 1.0$ and used pH 5.0 for $\Delta I = 0$ (16), then using Fig. 1, one can estimate that $\Delta I = 0.5$ corresponds to a pH value near 6.2.

The increase in fluorescence intensity can be observed visually as shown in Fig. 4. Fig. 4$A$ and $B$ show the increased emission from endocytic vesicles upon addition of 50 mM methylamine after a 15-min incubation in F-$\alpha_2$M. Fig. 4$C$ and $D$ show that a similar increase can be observed after a 5-min incubation, indicating that the endocytic vesicles are acidic within 5 min or less. Almost no bright spots could be observed on these cells before addition of methylamine.

## pH Dependence of $\alpha_2$M and EGF Binding

The pH dependence of $\alpha_2$M and EGF binding is shown in Fig. 5. The pH dependence of EGF binding has been described previously (7). As seen in Fig. 5, both ligands bind poorly
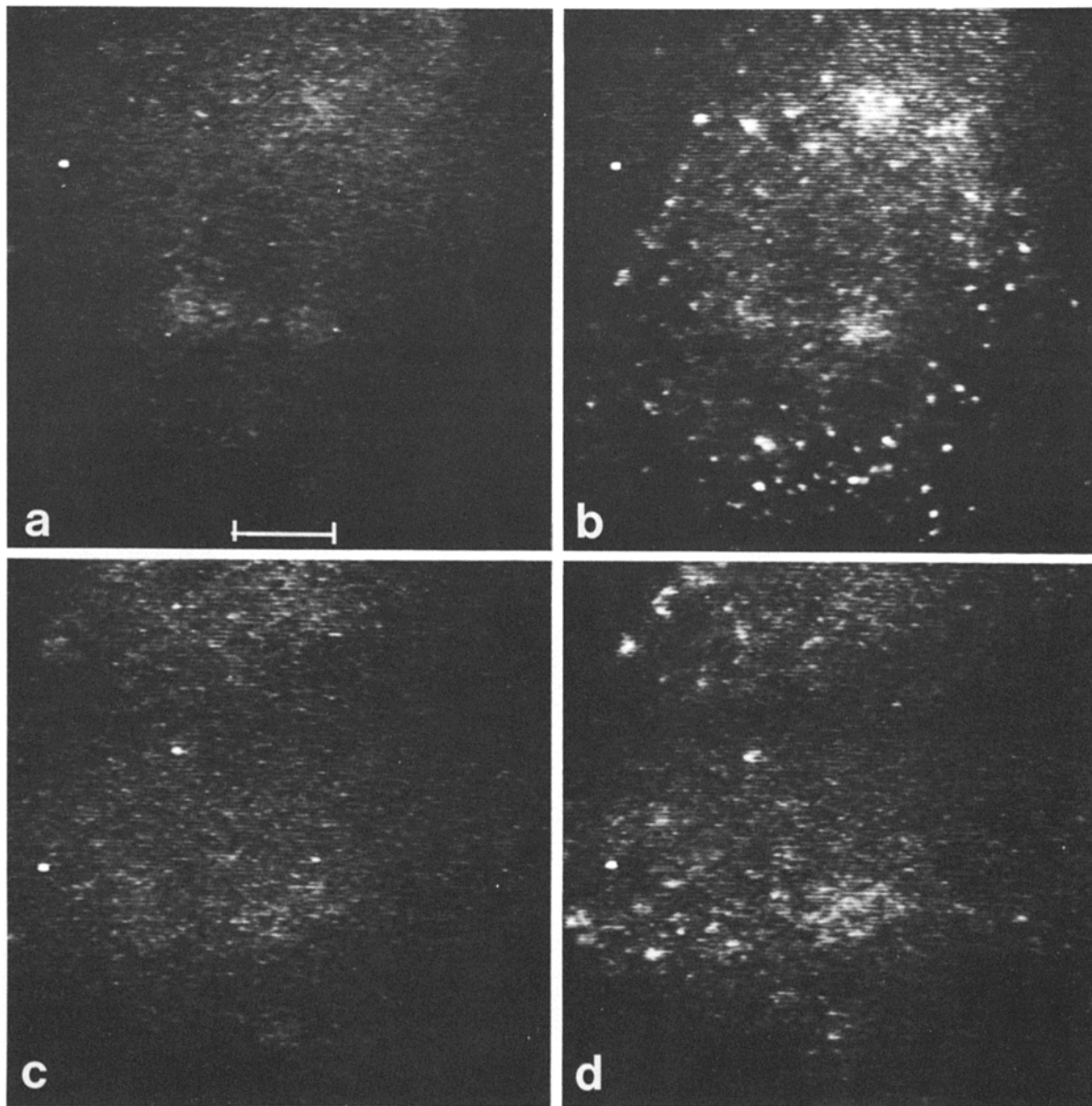


FIGURE 4   Effect of methylamine on fluorescence emission from endocytic vesicles. ($A$, $B$) Cells were incubated with F-$\alpha_2$M (100 $\mu$g/ml) for 14 min at 37°C. The cells were rinsed with measuring buffer, and placed on the microscope. A single cell is shown before ($A$) and 1 min after ($B$) addition of 50 mM methylamine to the measuring buffer. The plane of focus has been changed slightly between the two images because it was difficult to find bright spots to focus on before addition of methylamine. ($C$, $D$) Same as ($A$, $B$), but cells were incubated with F-$\alpha_2$M (300 $\mu$g/ml) for 5 min at 37°C. $C$ shows two cells observed 7 min after the start of the incubation, and $D$ shows the same cells 1 min later in the presence of 50 mM methylamine. Under these conditions, 80% of the fluorescein fluorescence could be competed by unlabeled $\alpha_2$M (5 mg/ml). Bar, 10 $\mu$M. $\times$ 1,500.
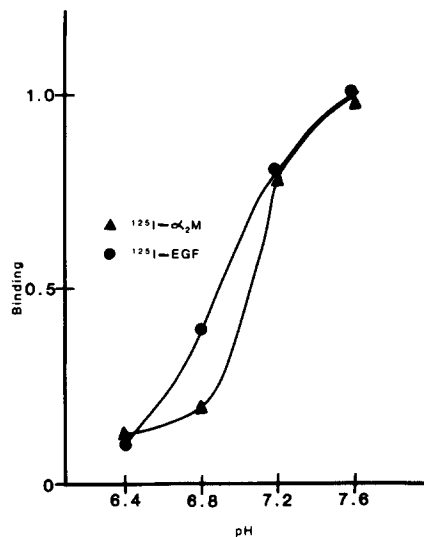
FIGURE 5  pH dependence of $\alpha_2$M and EGF binding. Cells were incubated at the indicated pH with $^{125}$I-$\alpha_2$M (3 nM) or $^{125}$I-EGF (2 nM) on ice as described in Materials and Methods. At the end of the incubation, cells were rinsed with ice-cold binding medium, dissolved in 1 N NaOH, and the cell-associated radioactivity was determined. The data shown are corrected for noncompetible binding. For $\alpha_2$M, the binding at pH 7.6 was 57 ng/mg cell protein, and for EGF the binding at pH 7.6 was 0.45 ng/mg cell protein.

below pH 6.8. Addition of agents which raise the vesicle pH above 6.8 would be expected to reduce the dissociation of ligand-receptor complexes.

## DISCUSSION

We have developed a microspectrofluorometric method to measure the pH of endocytic vesicles which contain F-$\alpha_2$M. We have previously shown that in BALB/c 3T3 fibroblasts these vesicles have a pH near 5.0 after a 15-min uptake and a 5-min rinse. By observing the increase in F-$\alpha_2$M fluorescence after treatment with methylamine (Fig. 4) it is now clear that endocytic vesicles are acidic after a 5-min incubation and a 2-min rinse. It is possible that endocytic vesicles become acidic almost immediately after formation. The rate of reacidification after removal of monensin (Fig. 2) indicates that the vesicles are capable of reaching pH 5 within 1 min or less. This rapid reacidification also indicates that the vesicles themselves contain a mechanism for regulating their internal pH.

The agents listed in Table I have all been reported to affect one or more steps in the receptor-mediated endocytosis pathway. The results reported in the literature often seem contradictory, and it is difficult to make generalizations about the interpretations of the observations. Since entry into acidic vesicles is an early step, it is possible that raising the vesicle pH could be responsible for some or many of the observed effects. The measurements reported here can serve as a basis for determining whether it is reasonable to consider an increase in endocytic vesicle pH as the cause of the observed effects.

Many enveloped viruses require an acidic pH for fusion with cell membranes and entry of the nuclear capsid (23). Several weak bases have been reported to block infectivity, and it has been suggested that this might occur by raising lysosomal pH (13). The concentrations of weak base required are generally consistent with the concentrations required to raise endocytic vesicle or lysosomal pH. However, monodansylcadaverine (500 $\mu$M) blocks influenza virus infectivity at concentrations which

do not raise vesicle pH. It has also been reported that monodansylcadaverine blocks endocytosis of vesicular stomatitis virus, and this inhibition was reported to occur by reducing the entry of virus into coated pits (23). This would be consistent with a mode of action for monodansylcadaverine other than raising pH.

"Nicked" diphtheria toxin can penetrate plasma membranes at pH 5.0 (25), and it has been shown that diphtheria toxin enters the same endocytic vesicles as $\alpha_2$M (26). Weak bases and monensin (12) are known to inhibit the toxicity of diphtheria toxin at approximately the concentrations which raise endocytic vesicle or lysosomal pH.

In the case of the enveloped viruses and diptheria toxin, either the endocytic vesicles or lysosomes could be the site of cytoplasmic penetration. Since the endocytic vesicles are the first step on the pathway, they would seem a likely candidate for the site of penetration. Virus particles or toxin chains which did not escape from the endocytic vesicles would be delivered to lysosomes where they might penetrate into the cytoplasm before degradation.

Many of the ligands which enter cells by receptor-mediated endocytosis are delivered to lysosomes, and many of the receptors are recycled to the plasma membrane. Several investigators have described the effects of the agents listed in Table I on various steps in the endocytosis pathway. The goal of these previous studies has been to identify the step in the pathway which is sensitive to these agents and, eventually, to understand the underlying basis for the sensitivity.

It is likely that at least some of the effects on endocytosis produced by the agents listed in Table I (except monodansylcadaverine) are due to an increase in endocytic vesicle pH. For example, it has been reported that ammonia blocks delivery of mannose-containing ligands to lysosomes in macrophages (4). Both monensin and chloroquine cause a loss of low density lipoprotein receptors from the cell surface, and this has been attributed to an inhibition of receptor recycling (9).

Monodansylcadaverine has been reported to block the endocytosis of a number of ligands, including epidermal growth factor (7), $\alpha_2$-M (24), 3,3',5-triiodo-2-thyronine (27), and vesicular stomatitis virus (24). It has also been reported to reduce the number of surface $\alpha_2$-M receptors (10). From our results, it is clear that at the concentrations used (20–500 $\mu$M), monodansylcadaverine is not acting as a weak base to raise endocytic vesicle or lysosomal pH. In several cases (1, 10, 13, 28), it has been suggested that dansylcadaverine produces effects similar to other weak bases. Either monodansylcadaverine and the weak bases produce similar effects by different mechanisms, or the effects of both are not related to the ability to raise the pH of acidic organelles.

There is growing evidence that exposure to an acidic environment is important for the processing of many ligands and receptors. Much of this evidence has been based on treatment of cells with agents which would be expected to raise the pH of acidic organelles. We have recently shown that the endocytic vesicles which are involved in uptake of many ligands are acidic. In this paper it has been shown that many of the agents which affect receptor-mediated endocytosis rapidly and reversibly raise endocytic vesicle pH at concentrations which affect endocytosis. These result are consistent with acidification of vesicles being an important processing step. It is important to realize, however, that these agents also may elicit effects at other sites, some of which are independent of their effects on pH.

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