pH Changes in Pinosomes and Phagosomes in the Ameba, *Chaos carolinensis*

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ABSTRACT Changes in pH are measured in pinosomes and phagosomes of single specimens of the giant, free-living ameba, *Chaos carolinensis*. Measurements of pH are made microfluorometrically, as previously described (Heiple and Taylor. 1980. *J. Cell Biol.* 86:885-890.) by quantitation of fluorescence intensity ratios (Ex_{489nm}/Ex_{452nm}, $Em_{520-560nm}$ from ingested fluorescein thiocarbamyl (FTC)-ovalbumin. After 1 h of pinocytosis (induced in acid solution), FTC-ovalbumin is found in predominantly small (≤ 5 μm in diameter), acidic (pH $\leq$ 5.0-6.2) vesicles of various shape and density. As the length of ingestion time increases (up to 24 h), the probe is also found in vesicles of increasing size (up to 100 μm in diameter), increasing pH (up to pH ~ 8.0), and decreasing density. Co-localization of fluorescein and rhodamine fluorescence, after a pulse-chase with fluorescein- and rhodamine-labeled ovalbumin, suggests vesicle growth, in part, by fusion. The pH in a single phagosome is followed after ingestion of ciliates in neutral solutions of FTC-ovalbumin. A dramatic acidification (Δ pH ≥ -2.0) begins within 5 min of phagosome formation and appears to be complete in ~20 min. Phagosomal pH then slowly recovers to more neutral values over the next 2 h. pH changes observed in more mature populations of pinosomes within a single cell may reflect those occurring within a single phagosome. Phagosomal and pinosomal pH changes may be required for lysosomal fusion and may be involved in regulation of lysosomal enzyme activity.

Little is known about the regulation of intracellular vesicle movements after internalization of macromolecules or particles by endocytosis (30). Quantitative information about the normal sequence of ionic changes within endosomes is needed to understand the regulation of endosomal events such as endosome-lysosome fusion and the degradation or protection of internalized substances. Until recently, no satisfactory quantitative technique existed for the continuous measurement of pH within specific subcellular compartments of a single, moving eucaryotic cell. The novel microfluorometric technique we designed and applied to cytoplasmic pH measurements in single motile amebae (11) is here extended to a study of pH changes in pinosomes and phagosomes of these amebae.

The free-living, fresh-water amebae, including *Chaos carolinensis*, are ideal cells for the study of a variety of fundamental processes. A great deal of quantitative information is available on both the physiology and morphology of free-living amebae (19) and extensive analysis of contractility and of endocytic mechanisms in these cells has been carried out over the last fifty years (7, 30). The exceptionally large size of *C. carolinensis* (up to 600 μm in diamenter) makes it ideal for micromanipulation and measurements of single cells.

This study follows changes in vesicle pH after induction of pinocytosis or phagocytosis of the pH-sensitive fluorescent probe, fluorescein-thiocarbamyl (FTC)-ovalbumin, by *C. carolinensis*. To measure pH changes we have employed a microfluorometric technique (10, 11) which permits measurements of fluorescence from pinosomes and from single phagosomes within single living cells. A preliminary report of this work has been presented (12).

MATERIALS AND METHODS

Materials

C. carolinensis, obtained from Carolina Biological Supply, Burlington, NC, are cultured in Marshall's medium (0.147 mM K_2HPO_4, 0.11 mM KH_2PO_4, 0.05 mM $MgSO_4$, 0.5 mM $CaCl_2$, pH 7.0) with mixed ciliates, as previously described (31). Where Marshall's medium (pH 4.0) is used, the composition is as follows: 0.26 mM KH_2PO_4, 0.05 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.14 mM KCl, pH 4.0 (slight pH adjustments with 0.1 M HCl are performed at 4°C). Addition of KCl brings the $[K^+]$ to that of normal Marshall's, deemed necessary because membrane

potential, streaming velocity and pinocytic activity of amebae are affected by $[K^+]$ (4, 5, 20). *Tetrahymena pyriformis*, also obtained from Carolina Biological Supply, are grown axenically in nutrient medium (1.5% Proteose peptone, 0.1% yeast extract, 0.2% dextrose). Samples for experimental use (or for supplemental feeding) are washed three times in normal Marshall's medium (centrifugation at 300 *g* for 5 min) before resuspension with or addition to test solutions.

FTC-ovalbumin is prepared by labeling chick ovalbumin (Worthington Biochemicals, Freehold, NJ, 2X crystallized) with fluorescein isothiocyanate (FITC), isomer I (Sigma Chemical Co., St. Louis, MO) according to established procedures (35). The dye-to-protein ratio of the FTC-ovalbumin used in these experiments is ~ 1.0 (using a molar extinction coefficient of 68,000 at 495 nm, pH 8.0, for bound fluorescein). Lissamine-rhodamine B sulfonyl (LRB)-ovalbumin is prepared by labeling chick ovalbumin (see above) with lissamine-rhodamine B sulfonyl chloride on celite (Molecular Probes, Plano, TX, as previously described [33]).

The pH of all solutions is measured with a VanLab Ag/AgCl Combination Microprobe Electrode (VWR Scientific, San Francisco, CA) on a Corning Model 10 pH meter (Corning Glass Works, Science Products, Div., Corning, NY) and adjustments are made with 0.1 M HCl or 0.1 M KOH. Glass-distilled water and reagent grade chemicals are used throughout this work.

Fluorescence Measurements

Spectral characteristics of FTC-ovalbumin in solution are measured both microfluorometrically and with a SPEX Fluorolog fluorometer (SPEX Industries, Metuchen, NJ). The microfluorometer used to quantitate fluorescence from small samples or from probe incorporated into single living cells has been described in detail elsewhere (10, 11). Briefly, on a Zeiss Photomicroscope, the intensity of emitted fluorescence (520–560 nm) is measured while the wavelength of excitation is rapidly and automatically interchanged between 489 and 452 nm (1.3 s per pair of measurements). For FTC-ovalbumin, the ratio of fluorescence intensity at these two different excitation wavelengths is dependent upon pH, is independent of pathlength and concentration (of probe or of unlabeled protein), and is not significantly affected by ionic strength, type of buffer (PIPES, HEPES, Tris-maleate, or phosphate buffer), Ca^{++} or Mg^{++} ions, K^+-acetate, $(NH_4)_2\,SO_4$, or photobleaching (10). To reduce overlap between the excitation and emission filter spectra, thereby reducing background fluorescence, the 496 nm excitation filter used in the earlier work has been replaced by a narrowband 489 nm interference filter (bandwidth at 50% T_{max}0.6nm; Spectro-Film, Inc., Winchester, MA). At a given pH, there is a linear relationship between the fluorescence intensity of the probe when excited at these two wavelengths (489 nm vs. 496 nm), and a simple conversion equation (obtained via linear regression analysis, data not shown) has been used to translate Ex_{489}:Ex_{452} values into the equivalent Ex_{496}:Ex_{452} values. Corrections are made for background fluorescence at each wavelength and calibration of these ratios with respect to pH is described in detail below. Measurements are made with a Zeiss 25X Pol Neofluar objective (N.A. 0.6) and an aperture 45 or 75 μm in diameter. Image intensification and recording is carried out as described elsewhere (33).

Induction of Pinocytosis and Phagocytosis

Pinocytosis or phagocytosis is induced in healthy specimens of *C. carolinensis*, starved for 48 h in Marshall's medium, in Marshall's medium containing FTC-ovalbumin. Pinocytosis is induced by rinsing starved amebae in Marshall's medium, pH 4.0, then immersing them in Marshall's medium, pH 4.0, with a final FTC-ovalbumin concentration of 0.3 mg/ml (all at room temperature [RT], ~22°C). At various times after immersion in this solution, amebae are removed, washed with a large excess of Marshall's medium, pH 7.0, RT, to remove adherent, noninternalized probe, and then are observed and measured immediately under these conditions in microscope chambers (clean glass cover slips with 250-μm Mylar spacers, Dupont Sorvall, Newton, CT). In some experiments, amebae are centrifuged using a slight modification of conditions previously shown both to stratify organelles in these cells (1) and to separate Alcian-blue-containing pinosomes into various classes (6). Amebae removed from the induction solution are immersed in a large volume of ice-cold Marshall's medium, pH 7.0, for 10 min, then are placed on 1 ml of a 10% sucrose cushion overlaid with 1 ml Marshall's medium, pH 7.0 (all at 0–4°C), and are centrifuged for 30 min at 1,500 *g* (4,000 rpm, 4°C, Sorvall RC-5 Superspeed centrifuge, SS-34 rotor). After centrifugation, amebae are carefully pipetted onto chilled microscope chambers in Marshall's medium, pH 7.0, 0–4°C. Care is taken at this point to keep track of the centrifugal and centripetal ends of the amebae. Measurements and observations of centrifuged amebae are made on a thermostatted (4–7°C) microscope stage (prototype unit: Cambion Corp., Cambridge, MA and Rainin Instrument Co., Boston MA), to inhibit redistribution of organelles. Cells recover completely when returned to room temperature.

In pulse-chase experiments, amebae are removed after 1–3 h of pinocytosis in FTC-ovalbumin, rinsed in Marshall's medium (pH 7.0, then pH 4.0), and reimmersed for various lengths of time in LRB-ovalbumin solution (0.3 mg/ml in Marshall's medium, pH 4.0). Samples of amebae are then removed and observed, or are treated and measured as described above.

Phagocytosis is induced by immersing a starved ameba in a 1:1 mixture of concentrated *T. pyriformis* (see Materials and Methods) in Marshall's medium and 3.0 mg/ml FTC-ovalbumin in 2.5 mM PIPES, pH 7.0, RT. The ameba is observed microscopically (with phase or Nomarski optics) in a cover slip chamber with 100-μm spacers until a single food cup successfully traps one of the ciliates and (apparently) seals to form a phagosome. This point is taken as zero time, and the chamber is immediately flushed with a large excess of Marshall's medium, pH 7.0, RT. The single, initially very large (up to 200 μm in diameter), fluorescent phagosome is very easy to locate and to measure for ~1½ h, after which its decreasing size and rapid movements within the cell make it increasingly difficult to find and follow (see Results).

RESULTS

Calibration of Fluorescence Intensity Ratios

The necessity for *in situ* calibration of pH-sensitive probes has been amply demonstrated and discussed in detail (11, 25, 34). To obtain a standard curve appropriate for calibration of signals from internalized probe, the following experiments are performed. An ameba which has pinocytosed FTC-ovalbumin for 12–24 h is washed in Marshall's, pH 7.0, 22°C, then is immersed in a pH-equilibration solution composed of 100 mM NH_4Ac, 10 mM Tris-maleate, pH 6.00, 6.50, 7.00, 7.50, or 8.00. One of these solutions is placed into a clean, drawn-out silica glass Pasteur pipette with an ameba. Positive pressure is applied to the wide end with a pipette bulb, forcing the ameba into the progressively narrower bore until the plasma membrane ruptures (32). The plasma-membrane-free cytoplasm, containing intact fluorescent pinosomes (see Fig. 1, *inset*), is incubated in the equilibration solution for 20 min by which time the fluorescence intensity ratio from the probe is observed to reach a new, stable value. The fragment of pipette containing the ruptured ameba is then transferred to a glass microscope slide, covered with mineral oil and a cover slip, and is measured microfluorometrically (the entire length of pipette containing fluorescent vesicles is sampled by a minimum of 10 measurements). The results are shown in Fig. 1, where average ratios from at least three amebae so treated are represented at each pH value tested. The fluorescence intensity ratios calibrated within vesicles are in excellent agreement with those previously determined for probe localized in the cytoplasm of intact cells (11). We have used the standard curve shown in Fig. 1 to calibrate fluorescence intensity ratios in the experiments reported here.

Pinocytosis

CONTINUOUS INGESTION OF FTC-OVALBUMIN

MICROSCOPIC OBSERVATIONS: After removal from the pinocytosis-inducing solution, amebae recover quickly from the rounded, rosette shape characteristic of ingestion, and begin to move, with rapid cytoplasmic streaming. Numerous fluorescent inclusions of various sizes are distributed randomly among other organelles throughout the cell. Measurements of pH in pinosomes are made on chilled, centrifuged cells since this procedure temporarily inhibits vesicle movement and separates vesicles according to size and density.[1]

[1] Endocytic vesicles or vacuoles are identified according to the nature of their origin: as pinosomes, if induced by formation of pinocytic channels around submicroscopic, soluble stimuli; and as phagosomes, if induced by food cup formation around a prey organism, without regard to subsequent fusion among themselves or with other

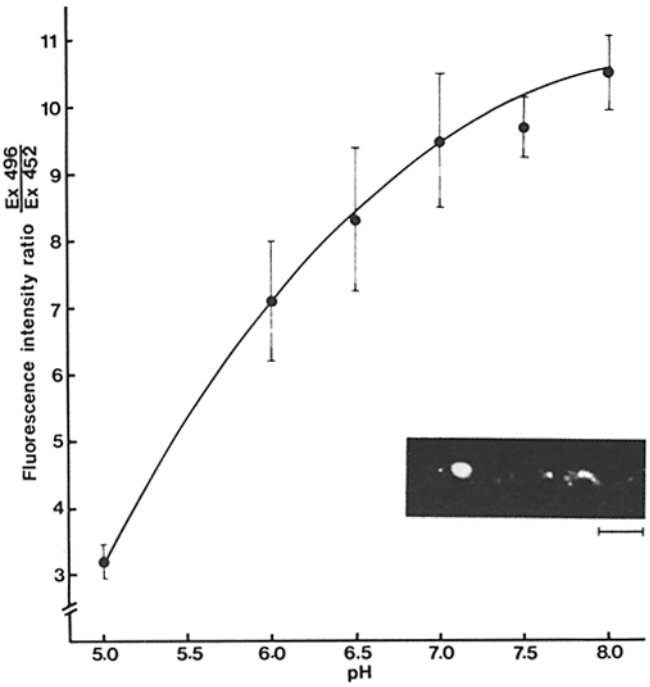

FIGURE 1 Standard curve of fluorescence intensity ratio (Ex_{496}/Ex_{452}, $EM_{520-560}$) vs. pH for FTC-ovalbumin in pinosomes. Fluorescence measurements are of pinosomes (from ≥ 3 amebae at each point (± SD), whose internal pH is fixed as described in the text. *Inset*: fluorescent pinosomes after rupture of an ameba within a capillary tube. Photograph from image intensifier. Bar, 100 μm.

Centrifugation of amebae results in stratification of organelles essentially as previously reported (1). It does not alter the number or morphology of organelles with respect to those of uncentrifuged cells. The distribution of FTC-ovalbumin-containing pinosomes in centrifuged amebae, as revealed by fluorescence microscopy of the living cells, is in many respects similar to that reported by Holter and Marshall (16) for fluorescently labeled γ-globulin, and to that reported by Chapman-Andresen (6) for Alcian-blue. Fluorescence and Nomarski micrographs of representative centrifuged amebae after 1, 5, and 24 h of pinocytosis of the probe are shown in Fig. 2. In the following descriptions, we shall refer to three regions of the centrifuged amebae, as marked in Fig. 2*b*: the light (or centripetal) end (Fig. 2*b* ▲), the middle region (Fig. 2*b* ○), and the dense (or centrifugal) end (Fig. 2*b* ●). After 1 h (Fig. 2*a*), fluorescence is distributed fairly randomly throughout the centrifuged amebae in small inclusions (many below the resolving power of the microscope), often of irregular shape. As the duration of pinocytosis increases, small fluorescent inclusions persist, which are concentrated in the dense portion of the centrifuged cells, (Fig. 2*b* and *c*). Furthermore, with increasing time, larger fluorescent vesicles appear (never at 1 h, but usually a few by 3 h), which concentrate into the light end of the cells when they are centrifuged (Fig. 2*b* and *c*). (Progressively larger fluorescent vesicles appear over time in uncentrifuged cells as well). Occasionally, vesicles of intermediate size are observed in the middle region, but at later time points this portion is usually free of fluorescence, as shown in these examples. This sorting process is gradual and continuous for up to ~15 h. The larger vesicles increase both in number and in size. Eventually, these lighter vesicles become extremely large (some > 75 μm in diamter) and usually also decrease in number after 15 h, suggesting formation via fusion of smaller vesicles and/or growth accompanied by excretion.

With Nomarski optics, small vesicles are difficult to identify. However, large fluorescent vesicles clearly correspond (Fig. 2*b* and *c*) to organelles that are morphologically indistinguishable from typical ameba food vacuoles (1). Free cytoplasmic fluorescence is never observed during the time course of these experiments.

If amebae are returned to normal Marshall's medium after these experiments, the number and fluorescence intensity of these inclusions gradually decreases, until only 1 or 2 very large, dim vesicles remain. These fluorescent vesicles can persist in subsequently unfed cells for 7 or more days.

PH MEASUREMENTS: The results of pH measurements obtained are summarized in Fig. 3. Each centrifuged cell is sampled with a minimum of ten measurements, and at least three amebae are measured at each time point. At early time points, or later in the dense end of the cells, small size and low pH (resulting in low fluorescence intensity) prohibit fluorescence quantitation from single pinosomes. (Indeed, many of these inclusions are below the resolving power of the microscope and appear as a fluorescent haze). Therefore, most measurements represent average pH of all the pinosomes included in the field of the measuring aperture. However, many measurements made at ≥ 8 h, in the light end of the centrifuged cells, represent single, extremely large vesicles with diameters >50 μ and pHs >7.25. Measurements are scored for relative position in the centrifuged cells, and averages of all the measurements in each region are plotted vs. time in Fig. 3. Clearly, as the duration of pinocytosis increases, some pinosomes become progressively less dense and more alkaline. At 1 h, when fluorescent pinosomes do not appear to be sharply stratified, measurements made in all three regions of the centrifuged cell appear similar (pH ~5.3). However, over the next 7 h, the measurements in the light end (Fig. 3, ▲) become progressively more alkaline, while average values from the dense end (Fig. 3, ●) remain about the same (pH ~5.2), and average values from the middle region (Fig. 3, ○) appear to become slightly more alkaline (up to ~5.4). By 13.5 h, all the measurements are more alkaline (dense region ~5.6, middle region ~6.4, light region ~7.6). Stratification of density, size and pH over time can be visualized by comparing the micrographs of typical centrifuged amebae (Fig. 2) to the corresponding time points in Fig. 3. In a given ameba, without exception, the pinosomes in the light end of the centrifuged cell are less acidic than those in the dense end and, as the duration of pinocytosis increases, the former become larger and more alkaline. Uncentrifuged cells also contain pinosomes of increasing size and pH as the duration of pinocytosis increases (data not shown).

PULSE-CHASE EXPERIMENTS

As described in Materials and Methods, some amebae are pulsed with FTC-ovalbumin, then chased with LRB-ovalbumin (data not shown). A typical cell pulsed for 1 h with FTC-ovalbumin and chased for 4 h with LRB-ovalbumin shows a distribution of fluorescein fluorescence and vesicle pH, after centrifugation, typical of a "5 h" cell: small vesicles (ave. pH 5.4) in the dense end, and two very large vesicles (one > 75 μm in diameter) in the light end with pHs of 7.2 (the larger one)

organelles (e.g. lysosomes and older food vacuoles). This classification is not meant to obscure or to minimize the importance of the latter events.

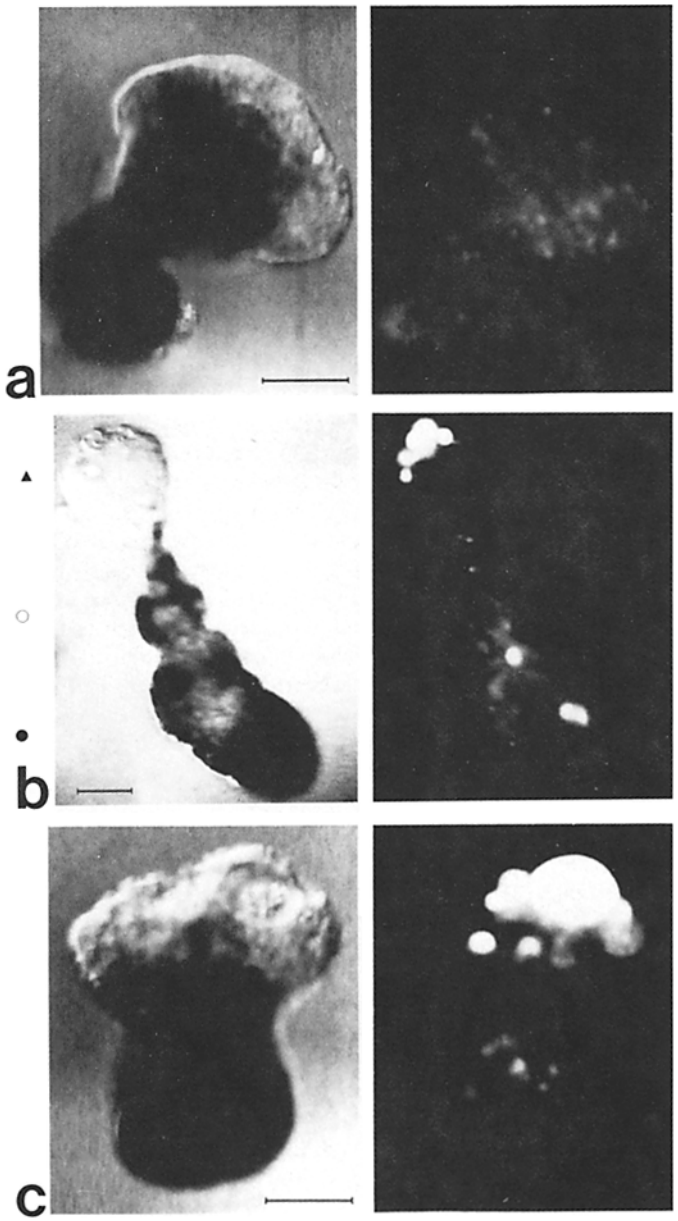

FIGURE 2 Nomarski (left) and fluorescent (right) images of centrifuged amebae after continuous pinocytosis of FTC-ovalbumin for 1 h (*a*), 5 h (*b*), and 24 h (*c*). In *b*, symbols identify the following regions of a centrifuged cell (▲) light end, (○) middle region, (●) dense end. Photographs from image intensifier. Bars, 100 μm.

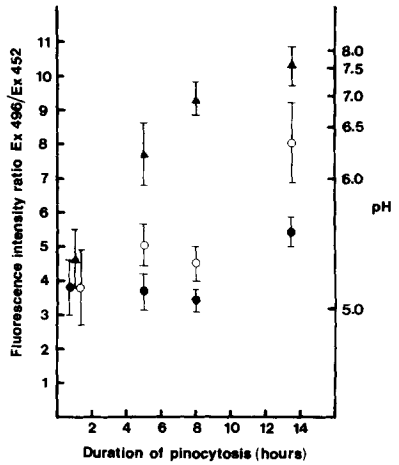

FIGURE 3 Average fluorescence intensity ratio and average pH vs. hours of pinocytosis for subpopulations of FTC-ovalbumin-containing pinosomes in centrifuged amebae. As defined in Fig. 3 and in the text: (●) dense end, (○) middle region, (▲) light end of cell. Bars represent SD in ratio units.

and 6.9. The rhodamine fluorescence in this cell is also confined to small vesicles predominantly in the dense end. At this stage, it is impossible to determine whether the rhodamine and fluorescein fluorescence occupy the same vesicles, though there is quite clearly no rhodamine fluorescence in the larger valuoles in the light end of the cell. If the chase time is extended to 24 h, the distributions of both types of fluorescent vesicles appear to match exactly. These distributions are fairly typical of a cell after 24 h of pinocytosis, in size, density, and pH. Notably, the largest vesicles observed (in the light end, one > 75 μm in diameter, pH 7.0), which resemble food vacuoles, contain both rhodamine and fluorescein fluorescence.

Phagocytosis

MICROSCOPIC OBSERVATIONS: After entrapment of ciliate and fluorescent probe, the phagosome is transferred to the tail of the ameba, as shown with Nomarski optics in Fig. 4 (top). The arrow indicates the location of the fluorescent phagosome, as shown in Fig. 4 (bottom). The fluorescent micrograph illustrates a common phenomenon—that of apparent "budding" or "micropinocytosis" of the phagosome (1, 5) or transfer of fluorescent contents away from the phagosome by some other mechanism (e.g., "pirhanalysis" [36]). This process typically begins ~30 min after vacuole closure, as

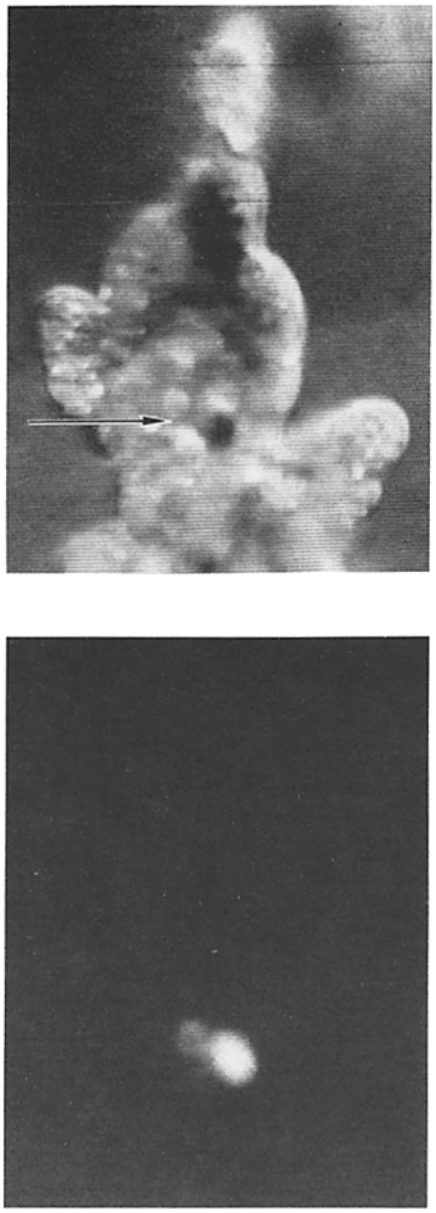

FIGURE 4 Phase contrast (top) and fluorescent (bottom) images of the tail region of a moving ameba ~30 min after phagocytic vacuole formation around a ciliate immersed in FTC-ovalbumin. Arrow (top) indicates vacuole corresponding to fluorescent vacuole (bottom). Photographs from image intensifier. Magnification ×200.

shown here, and often results in a rosettelike fluorescent vacuole. The resulting small fluorescent vesicles cannot be followed and measured in this cell type. They are quickly lost in the streaming endoplasm. In the appropriate plane of focus, the ciliate appears dark against the fluorescent background of the phagosome (not apparent in this image), and fluorescence is never observed within the ciliate. (If ciliates are mixed with FTC-ovalbumin more than 30 min before feeding the ameba, some fluorescent vesicles are observed in the ciliates themselves. Therefore, phagocytosis-inducing solutions are always freshly prepared immediately before use). During the first 30 min after phagosome formation, the vacuole decreases steadily in size until it is barely larger than the enclosed ciliate (~50 × 100 μm), and remains brightly fluorescent. At ~15–20 min the ciliate stops moving, and shortly after 30 min the phagosome breaks loose from its fixed position in the cortex of the ameba tail and enters the endoplasmic stream. After this time, the phagosome becomes increasingly difficult to locate, since it moves rapidly and progressive digestion of the ciliate renders the phagosome indistinguishable from other food vacuoles with phase-contrast or Nomarski optics. It can be observed that, eventually (>1 h), the phagosome begins to become larger again.

PH MEASUREMENTS: The change in pH over time in a typical single fluorescent phagosome is plotted in Fig. 5 Closure of the food cup, as determined by observation with Nomarski optics, is defined as zero time. The initial pH of the phagosome (~6.8) is, as expected, nearly that of the phagocytosis-inducing solution (pH 7.0). Phagosomal pH drops from this value to slightly less than pH 5.0 within the first 20 min after phagosome formation. After about 40 min at this low value, the pH begins to rise again and continues to rise for as long as the phagosome can be tracked (pH ~6.2 at 2.5 h in this experiment).

DISCUSSION

The data reported here show that pinosomes and phagosomes in *C. carolinensis* undergo a concerted sequence of size and pH changes as internalized material is processed through the cellular digestive system. As pinosomes mature (≥ 1 h), they become relatively less dense and more alkaline (Figs. 2 and 3). Measurements from the single, larger phagosome, induced at neutral pH, yield important information about earlier events after internalization of probe. The latter measurements (Fig. 5) reveal a rapid acidification of the phagosome to about pH 5.0, beginning almost immediately after internalization of probe. After ~40 min at this acidic value, phagosomal pH rises continuously for as long as it can be measured. At the latest time recorded, 2.5 h after phagocytosis of ciliate and probe from neutral medium, this value has reached pH 6.2, which corresponds well to pH values measured from pinosomes of comparable age (data not shown).

Interpretation of Fluorescence Intensity Ratios

After internalization of probe via pinocytosis (Fig. 2) or phagocytosis (Fig. 4), washing effectively removes all surface-

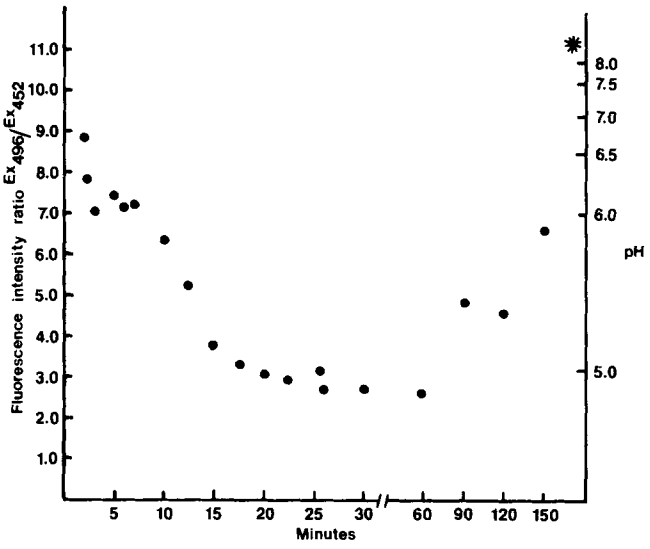

FIGURE 5 Fluorescence intensity ratio and pH vs. time (min) for a single fluorescent phagosome (as in Fig. 4). T_0 = vacuole closure, aperture diameter 45 μm, (*) value obtained upon incubation of the ameba in 10 mM Tris-maleate, 50 mM $(NH_4)_2SO_4$, pH 8.01 for ~5 min. The fluorescence intensity reaches this new stable value.

associated fluorescence (cf. reference 5). Detectable fluorescence remains confined to vesicles morphologically indistinguishable from the normal constituents of the ameba digestive cycle (1) and free cytoplasmic fluorescence is never observed (see Fig. 2). This observation suggests that, for at least 24 h, the fluorophore remains attached to an impermeant fragment of ingested protein and that subsequent experimental manipulations do not rupture vesicles.

Concentration, proteolytic degradation, and temperature changes may alter the fluorescent properties of FTC-ovalbumin. As long as the fluorophore remains attached to an intact ovalbumin molecule, it is prevented by the size of the protein molecule from self-quenching. That is, in an aqueous solution of the probe, with a dye/protein ratio of ~1:1 as used here, few, if any, fluorescein molecules will be close enough to self-quench (3.0–3.6 Å; reference 3). Even if the protein molecules are close-packed, most fluorophores will be separated by the diameter (~50 Å) of the protein. In vitro studies show that the fluorescence intensity ratio of FTC-ovalbumin, as used here, is independent of probe concentration (0.1–1.5 mg/ml) and is unaffected by the presence of up to 100 mg/ml unlabeled ovalbumin (10).

The possibility of alterations in fluorescence properties upon degradation of the probe must also be considered. However, qualitatively similar results have been obtained using a variety of absorbant pH-sensitive indicator dyes (18, 22, and Heiple and Taylor, manuscript in preparation). Thus, the measured pH drop accompanying decreased vacuole or vesicle size (therefore increased probe concentration) is not simply a trivial consequence either of concentration quenching or of the buffering capacity of the protein (pI ovalbumin = 4.53). Furthermore, generation of the standard curve from vesicles containing FTC-ovalbumin (Fig. 1) and alkalinization of fluorescent phagosomes upon treatment of amebae with alkaline solutions of ammonia (Fig. 5, asterisk), demonstrate that the probe can still respond to changes in pH with appropriate changes in fluorescence intensity. The fluorescence intensity ratios thus obtained, from pinosomes or from phagosomes, correspond well to those obtained from freshly injected, cytoplasmically localized probe (11), suggesting that proteolysis, if it has occurred, has not detectably altered the pH-sensitivity of the remaining probe.

Control experiments have shown that the fluorescence intensity ratio of cytoplasmically localized FTC-ovalbumin is not sensitive to changes in temperature from 4 to 22°C (10). Though the effect of low temperature on the pH of a particular pinosome *in situ* remains to be determined, the equivalent distribution of pH values among pinosomes of amebae at 4°C and 22°C suggests that significant pH changes are not induced at low temperature, and pH differences among pinosomes at 4°C clearly persist.

Distribution of Fluorescent Pinosomes in Centrifuged Cells

Comparison of sizes and of pH of pinosomes in centrifuged and uncentrifuged amebae reveals no significant differences. Centrifugation has been used extensively to study distribution of organelles in these cells (e.g., see references 1, 6, 16) and does not appear to induce artifacts at the centrifugal forces used here. The distribution of fluorescence among pinosomes of various size does differ somewhat from previous descriptions (6, 16, 23). Notably, large pinosomes (or vacuoles derived from them), which centrifuge to the lighter half of the cell, have not been reported previously. Technical differences such as nature of inducer, duration of pinocytosis, centrifugal force used or technique of observation are probably responsible for this discrepancy. The cause of this progressive decrease in density of the pinosomes is unknown.

The coexistence, within single mature pinosomes, of fluorescein and rhodamine fluorescence, after pulsing with FTC-ovalbumin and chasing with LRB-ovalbumin, suggests that the mechanism of increase in pinosome size may include fusion of pinosomes with one another. Fusion among pinosomes, food vacuoles, and preexisting acid phosphatase-positive granules in these amebae has been previously reported (2, 5, 6).

pH Changes in Pinosomes and Phagosomes

Measurements of pH in pinosomes of free-living amebae have not been previously reported. However, some indirect evidence for pH changes in pinosomes of *C. carolinensis* is given by the post-internalization release of bound ferritin (pI 4.4) from the glycocalyx of the pinosome (23). In an elegant qualitative study of pH changes in food vacuoles (phagosomes) of *Ameba proteus* and of *Ameba dubia*, after phagocytosis of ciliates stained with absorbant indicator dyes, Mast (22) describes an initial decrease, during the first 15 min, in phagosomal pH (usually to no less than 5.6) followed by gradual alkalinization (to ~7.6) over the next several hours. This time course of pH change agrees well with that reported here. A similar endosomal acidification has also been observed in mammalian phagocytes after challenge with absorbant indicator dye-stained yeast or bacteria (e.g., see references 17, 18, 21) or with fluorescently labeled stimuli (9, 24, 29). These investigators report minimum pH values ranging from 3.0 to 6.5, depending on the cell type, stimulus, and indicator dye used.

In mammalian cells, alkalinization of the phagosome subsequent to its acidification has not been observed. Recently, however, using fluorescently labeled bacteria, Segal et al. (29) have documented a slight, transient phagosomal alkalinization, occurring ~2 min after phagosome formation in normal human leukocytes. A transient phagosomal alkalinization has also been observed in normal mouse peritoneal macrophages (9) after challenge with immunofluorescently labeled yeast.

Both the cause(s) and effect(s) of endocytic vesicle pH changes remain obscure. Acidification of an endocytic vesicle may reflect fusion with acidic lysosomes (e.g., see references 13, 27); however, there is no direct evidence for this. Some investigators (26) report the appearance of lysosomal marker enzymes in leukocyte phagosomes well before acidification is observed, whereas others (28) find that marker enzymes do not appear in these organelles until acidification is well on its way. Histochemical localization of acid phoshatase activity (optimum pH 4.5, [15]) in centrifuged, fixed specimens of *C. carolinensis* reveals a distribution corresponding to that of Alcian blue-containing pinosomes (8). However, activity, presumably via fusion with lysosomes, is not seen within phagosomes until they are ~1 h old. Although these data must be interpreted cautiously, due to the possibility of fixation artifacts, they nevertheless suggest the possibility that the phagosomal pH drop that we have measured (~2.0 U within 20 min) is complete well in advance of the appearance of detectable lysosomal enzyme activity. Alternative mechanisms for endosomal acidification have not been proposed. Since a Donnan equilibrium across the ameba glycocalyx can result in a glycocalyx pH 1.3 units lower than that of the medium (14), simple concentration of the vesicle contents could result in a rapid drop in endosomal

pH. Endosomal acidification is probably not responsible for ciliate death in ameba phagosomes (22), and its function remains to be determined. Sequential activation of degradative enzymes by the continuously changing phagosomal pH has been suggested in leukocytes (18). Segal et al. (29) propose that the transient alkalinization they observe in leukocyte phagosomes facilitates bacterial killing, as it is absent from diseased cells with impaired bactericidal function.

Phagosomal and pinosomal pH changes may regulate fusion with lysosomes as well as provide an optimal pH for lysosomal enzyme activity after fusion. It remains possible that acidification and fusion are functionally independent events, in spite of their apparently close spatial and temporal relationship. Dissection of these events, and their relationship to intracellular sorting of internalized substances, will clearly require increased spatial and temporal resolution in techniques used to study these processes. The work reported here represents our initial efforts to meet this challenge. Our microfluorometric technique renders possible, for the first time, the continuous quantitation of pH within a single phagosome, *in situ*, as it progesses through the digestive cycle of the ameba. This approach also permits the simultaneous, detailed correlation of pH changes with other cellular events. Having defined the normal sequence of pH changes in ameboid phagosomes and pinosomes, we are now extending these studies to address the questions raised above, in this and other cell types, using a more sophisticated microfluorometer.

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