

Acidification of Phagosomes Is Initiated before Lysosomal Enzyme Activity Is Detected

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ABSTRACT We have measured changes of pH in a protein's microenvironment consequent on its binding to the cell surface and incorporation into pinosomes. Changes of pH were measured from single, living cells and selected regions of cells by the fluorescence ratio technique using a photon-counting microspectrofluorimeter. The chemotactic agent and pinocytosis inducer, ribonuclease, labeled with fluorescein (FTC-RNase), adsorbed to the surface of *Amoeba proteus*, and was pinocytosed by cells in culture media at pH 7.0. The FTC-RNase entered an apparently acidic microenvironment, pH \sim 6.1, upon binding to the surface of amoebae. Once enclosed within pinosomes, this protein's microenvironment became steadily more acidic, reaching a minimum of pH \sim 5.6 in <10 min. FTC-RNase pinocytosed by the giant amoeba, *Chaos carolinensis*, entered pinosomes whose pH was correlated with their cytoplasmic location during the initial 30–40 min after pinocytosis. The majority of pinosomes containing FTC-RNase clustered in the tail ectoplasm of *C. carolinensis* during this interval and had a pH of \sim 6.5; those released into endoplasm and carried into the tip of cells had a pH below 5.0. As pinosomes became distributed at random in *C. carolinensis* (1–2 h after initial pinocytosis), differences in pH between tip and tail pinosomes vanished.

We have also measured the pH within single phagosomes of *A. proteus*. Phagosomal pH dropped steadily to \sim 5.4 within 5 min after particle ingestion in 70% of the cells measured, and reached this level of acidity within 10 min in 90% of the cells measured. By contrast, stain for the lysosomal enzyme, acid phosphatase, was evident within only 20% of 5-min-old phagosomes visualized by light microscopy, and within only 40% of 10-min-old phagosomes. A microfluorimetric assay was used to simultaneously record changes in pH, and the initial deposition of lysosomal esterases, within phagosomes of single, living *Amoeba proteus*. Near complete acidification of the phagosome was recorded from some cells before phagosomal fusion was evident by this microfluorimetric assay. From other cells, however, continued acidification of phagosomes was recorded after lysosomal fusion was initiated. We conclude that acidification of phagosomes by *A. proteus* is initiated but not necessarily completed prior to phagosome-lysosome formation, and that the two events are closely linked in time. Initial acidification of endosomes is a property intrinsic to the plasma membrane which envelops particles at the cell surface, rather than the result of lysosomal fusion with phagosomes.

Binding of appropriate ligands to the cell surface can initiate many cellular responses including chemotaxis. The free-living amoebae exhibit chemotaxis in gradients of some basic proteins such as ribonuclease (Our unpublished observations, and reference 16). These same proteins induce adsorptive pinocytosis when applied uniformly to amoebae, a response characteristic of other chemotactic cells (35).

Once endocytosed, many substances are degraded by lyso-

somal enzymes. These enzymes are delivered into the endosome by fusion of its membrane with the membranes of 1^o and 2^o lysosomes. However, not all particles and molecules endocytosed are subsequently digested. Many symbiotic and parasitic organisms can, for example, prevent fusion of lysosomes with the endocytic vesicles containing them, and can, as a result, avoid digestion while living within host cytoplasm (7, 13, 17). Furthermore, there are severe restrictions on the

fusion of lysosomes with cellular membranes other than those of newly formed endosomes. Healthy organelles would otherwise be needlessly destroyed, and an excess of lysosomal enzymes delivered into the aging endosome.

It is not presently known how lysosomal fusion with endosomes is regulated. Answers to this fundamental question may depend on well-defined spatial and temporal characterizations of the cellular, macromolecular, and ionic events leading up to fusion of lysosomes with endosomes. Changes in the pH of endosomes occur soon after initial endocytosis, and may therefore have relevance to the problem of endosome-lysosome fusion. Numerous studies since Metchnikoff (20) confirmed that initially phagosomal contents are acidic and that subsequently they may return to near neutral pH (6, 14, 15, 19). Using the fluorescence ratio technique (23), it has recently been possible to accurately quantify pH changes within endosomes over time. Thus, rapid drops in phagosomal pH have been recorded within newly formed phagosomes of the amoeba, *C. carolinensis* (9), and within the phagosomes of macrophages (6). Less is known about the kinetics of phagosome-lysosome fusion, because no techniques have been available to quantitatively measure this event in the living cell.

The kinetics of pH changes within pinosomes have not been recorded with good temporal resolution. However, Heiple and Taylor (9) showed that a subpopulation of pinosomes in *C. carolinensis* was acidic 1 h after initial endocytosis and Tycko and Maxfield (32) showed that pinosomes of fibroblasts are acidic at 20 min after initiation of pinocytosis.

We have employed a photon-counting microspectrofluorimeter, improved from a previous design (8, 10), to record the pH within pinosomes of single *A. proteus* as soon as 5 min after initial endocytosis, and for up to 24 h thereafter. We have, moreover, measured the pH at the surface glycocalyx of amoebae, to which chemotactic proteins adsorb prior to their uptake by pinocytosis. Temporal records were also made of the pH within pinosomes of the giant amoeba, *Chaos carolinensis*, and, in addition, we characterized differences of the pH within pinosomes correlated with their initial location after uptake in this cell. Finally, we recorded the early (2.5–20 min) time course of pH changes within phagosomes of single *A. proteus*, and, in parallel, determined the time course by which lysosomal enzyme activity is detectable within the newly formed phagosomes of *A. proteus*, both by conventional light- and electron-microscopic cytochemistry as well as by a microfluorimetric assay used to study phagosome-lysosome fusion in single, living cells.

MATERIALS AND METHODS

Cells: *A. proteus* and *C. carolinensis* (Connecticut Valley Biological Supply Co., Southampton, MA) were cultured in the following medium: 0.3 mM $K_2HP_4O_6$, 0.3 mM KH_2PO_4 , 0.08 mM $CaCl_2$, 0.08 mM KCl , 0.008 mM $MgSO_4$, and 0.17 mM $NaCl$, adjusted to pH 7 with 0.1 N HCl or 0.1 N KOH . Amoebae were fed as previously described (24) three times weekly with *Tetrahymena pyriformis* (Carolina Biological Supply, Burlington, NC) grown axenically in 1.5% protease peptone, 0.1% yeast extract, and 0.2% dextrose. Amoebae were starved for ~48 h before all experiments.

Protein Labeling with Fluorescein: Chick ovalbumin, 2 times crystallized (Worthington Biochemicals, Freehold, NJ) and ribonuclease-A (fraction III, Sigma Chemical Co., St. Louis, MO) were labeled with fluorescein isothiocyanate isomer I (Sigma Chemical Co.) according to published methods (33). The dye-to-protein ratio of FTC (fluorescein¹)-ovalbumin was 1; for FTC-ribonuclease it was 0.2.

¹ **Abbreviations used in this paper:** CFD, carboxyfluorescein diacetate; FTC, fluorescein; FTC-RNase, ribonuclease labeled with fluorescein.

Microspectrofluorimetric Measurement of Fluorescence Intensity Ratios: Fluorescence intensity ratios ($E_{\lambda_{489}}/E_{\lambda_{452}}$) from single cells containing FTC-labeled proteins or from microcapillary tubes containing the proteins were measured with a microspectrofluorimeter based on the design of Heiple and Taylor (10). As shown previously with their apparatus, this ratio of fluorescence intensity is highly dependent on pH in the range 5 to 7, but is not affected by the ionic strength or buffering species in the solvent, or by photo-bleaching (10). Further details specific to the individual experiments are given in the figure legends.

The present microspectrofluorimeter was improved in four areas to increase both spatial and temporal resolution (see Fig. 1):

Excitation beam. The heat-filtered beam was gated by a filter holder driven by a stepping motor (Superior Electric Co., Bristol, CN), controlled by a Rockwell AIM-65 microcomputer (Rockwell International Corp., Newport Beach, CA). This gate blocks the excitation beam completely or selects one of the two excitation wavelengths ($E_{\lambda_{452}}$ or $E_{\lambda_{489}}$) by swinging the appropriate excitation filter (10-nm bandwidth, Spectro-Film, Inc., Winchester, MA) into the light path. The three-position gate prevents bleaching of fluorescent proteins within cells by the excitation beam before and after the experiment, but allows rapid (10–110 ms programmable) switching to excitation filters. The stepping motor is remotely controlled by the microcomputer (Fig. 1 b) through a slow-syn translator. Excitation beam intensity is adjusted by a set of Zeiss neutral density filters. The microscope and filter assembly have separate vibration-damped supports.

Emission beam. Light from the photometer head was gated by an electro-mechanical shutter (22512AOX5, Vincent Associates, Rochester, NY) driven by the microcomputer through a power supply energizer (100-2, Vincent Associates), and collected by a 3.18-mm-diam light guide (randomized fiber arrangement, Galileo Electro-Optics, Sturbridge, MA), which delivers the light to the photomultiplier.

Photon counter. Instead of the analog-to-digital conversion of Heiple and Taylor (8, 10), sensitivity and signal-to-noise ratio were maximized by reducing various forms of excess noise through photon counting (1). The RCA8852 end-window multi-alkali photomultiplier tube (Bertan Associates, Syosset, NY) was operated at 2,000 V direct current, and housed in a water-cooled thermoelectrically-refrigerated chamber, which is radio-frequency shielded and maintained at $-30^\circ C$ (TE-104-RF, Products for Research, Inc., Danvers, MA). A set of defocusing magnets (PR-411, Products for Research, Inc.) was placed inside the cooling chamber to decrease the effective photocathode to ~15 mm in diameter to match the emission beam, which falls within a spot of ~10 mm in diameter on the photocathode. The anode current was fed into model 631 pulse amplitude discriminator (EMR Photoelectric, Princeton, NJ). The frequency of the pulses from the photomultiplier was preselected before counting by a "versatile interface adapter" chip in the AIM-65 microcomputer (4), giving a linear response up to 100,000 counts/sec.

Supervising control. The AIM-65 synchronized stepping motors, shutters, and photon counter, and provided statistical analysis of the data.

These improvements have resulted in much better spatial and temporal resolution. Measurements may be made in regions as small as 20 μm in diameter for relatively stationary cells. The time required for a single measurement has been cut down to 300 ms (Fig. 1 b). Excitation intensity has been reduced to the point that no autofluorescence was measured from the prepinocytotic amoebae.

Pinocytosis and Phagocytosis: Fluorescent pinosomes were formed when amoebae were immersed at 22°C for 2 to 20 min in culture medium, pH 7.0, containing 0.1 mg/ml ribonuclease labeled with fluorescein (FTC-RNase). At the end of this period, FTC-RNase bound to the glycocalyx was removed by four washes in culture medium pH 10.7 followed by two washes in culture medium pH 7.0. These washes were accomplished in <2 min by pipetting the amoebae from one pool (2 ml) to another of six washes on a plastic petri dish viewed under a dissecting microscope. In cells not given time to pinocytose (immersed for <10 s in the FTC-RNase before washing), such washing removed all but 0.1 to 0.3% of surface bound FTC-RNase from *A. proteus* and all but 0.5 to 1.0% of it from *C. carolinensis*. Washed cells containing pinocytosed FTC-RNase were transferred for microspectrofluorimetric measurements to microscope chambers consisting of a coverslip supported on a slide by clay feet and containing culture medium pH 7.0. Initiation of pinocytosis was defined as time zero in all measurements, but formation of pinocytotic channels and pinosomes is not immediate, so this definition gives a maximum age for the pinosomes. Phagosomes containing living *Tetrahymena* and FTC-ovalbumin ingested together in culture medium at pH 7.0 were induced in *A. proteus* as previously described for *C. carolinensis* (9). We took apparent sealing of the phagosome as time zero.

Detection of Lysosomal Enzymes within Phagosomes by Light and Electron Microscope Cytochemistry: Stain for the lysosomal enzyme acid phosphatase was deposited in the lysosomal compartments of *A. proteus* by a modification of Gomori's classical method (3). Several

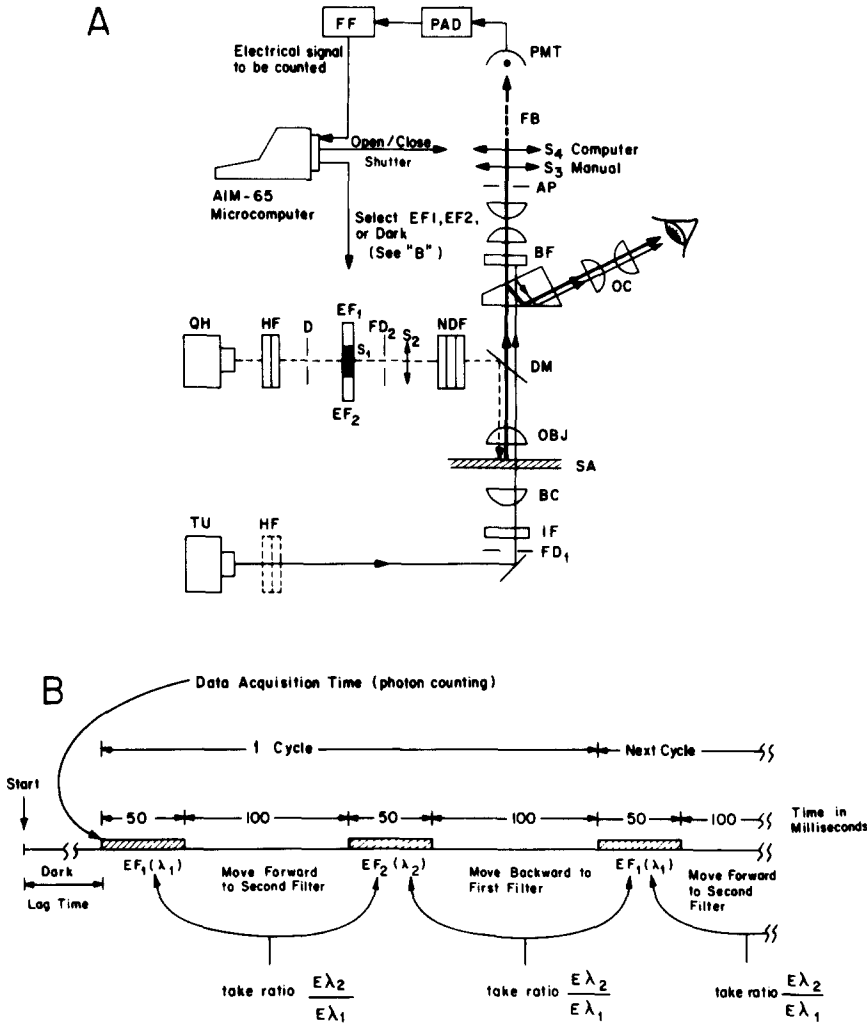


FIGURE 1 (A) Schematic representation of the microfluorimeter used to quantify fluorescent signals. The dotted line represents the epi-excitation light from a quartz-halogen lamp (QH), the heavy solid line represents the path of fluorescent light from the sample (SA), and the lighter solid line represents light from a substage tungsten lamp (TU). A substage interference filter (IF) selects a wavelength of light, for bright-field observation, that will not pass through the barrier filter (BF) to the photomultiplier (PMT). The fluorescent filters, EF1 and EF2, select the wavelength of the excitation light, and S₁ blocks the light beam. Other symbols are as follows: HF, heat filter set; FD₁ and FD₂, field diaphragms; DM, dichroic mirror; OBJ, objective; BC, bright field condenser; OC, ocular; D and AP, variable apertures; PAD, pulse amplitude discriminator; FF, prescribing flip-flops; NDF, neutral density filter; FB, fiber bundle. (B) Timing diagram for operation of the stepping motor and data acquisition. The time scale is given on the horizontal axis. Shaded areas signify the duration of photon counting (S₃ and S₄ are open). When the operation is started, either manually or automatically, there is a short, programmable lag time (10 to 100 ms). Then data acquisition takes place in a periodic fashion. One complete cycle only is shown in the diagram. In each cycle, photon counts (selected through a 520–560-nm barrier filter, BF) are integrated over 50 ms for each excitation filter (EF₁ or EF₂). The ratio of these two sums (EF₂/EF₁) is computed twice per cycle.

thousand amoebae, settled on the bottom of plastic petri dishes, were presented with 100 times their number of living *Tetrahymena*. Within 2 min after this presentation, 5–10% of the amoebae had phagocytosed one *Tetrahymena* and were firmly adherent to the dishes. Those *Tetrahymena* not taken up during 2 min were removed from the dishes by four rinses of culture medium. The time of removal of excess *Tetrahymena* was taken as time zero, though obviously phagocytosis may have been completed as much as 2 min earlier. At selected intervals after phagocytosis, all but a residue of culture medium was aspirated from the dishes, to which were added 2 ml of 2.5% paraformaldehyde and 4.5% glutaraldehyde in 200 mM cacodylate buffer, pH 7.0. Amoebae were fixed for 30 min at 22°C, washed three times by centrifugation at 100 g for 2 min in 200 mM Tris-maleate buffer, pH 5.0, and incubated for 15 min at 37°C in a filtered Gomori stain, pH 5.0, that had been warmed for 1 h to 37°C. The modified Gomori stain contained: 10 ml of 1.25% Na-β-glycerolphosphate, 10 ml of glass-distilled H₂O, 10 ml of 200 mM Tris-maleate buffer, and 20 ml of 0.2% lead nitrate, which was added dropwise to the previous components. Controls for nonspecific staining contained 0.2 M NaF, in addition to the above ingredients. After staining, amoebae were washed with 100 mM cacodylate buffer pH 7.0, 1% acetic acid, and again with buffer. Amoebae to be examined by light microscopy were immersed in 1.0% ammonium sulfide for 2 min, washed once with glass distilled H₂O, and mounted in chambers on glass slides. The presence or absence of the black stain in phagosomes of amoebae were scored single blind. Cells for electron microscopy were postfixed (without immersion in ammonium sulfide) in 1% OsO₄ in 150 mM cacodylate, pH 7.0, for 20 min at 4°C, dehydrated in ethanol and propylene dioxide, and embedded in Spurr's resin (27). Sections were examined and photographed on a Philips 300 electron microscope without further staining.

Stain for esterases was deposited in the lysosomal compartments of *A. proteus* by a modification of Shnitka and Seligman's method (26). Amoebae that had phagocytosed heat-killed *Tetrahymena* (as above for Gomori staining) were fixed for 2 h in 10% (vol/vol) formalin and then stained with a solution of naphthol-AS acetate (Sigma Chemical Co.) and fast blue salt BB (Sigma Chemical Co.) as described (26), but without NaF added, for 15 min at room temperature.

Detection of Lysosomal Esterases within Phagosomes of

Living Cells by a Microfluorimetric Assay: Living, washed *Tetrahymena* were suspended in a test tube in 4 ml of amoeba's culture medium, immersed for 10 min in a boiling water bath, and washed twice in 10 ml of culture medium. At least 10 min before the initiation of phagocytosis, the washed pellet of heat-killed *Tetrahymena* was suspended in 4 ml of culture medium (pH readjusted to 7) containing 0.06 mg/ml of 5 [6] carboxyfluorescein diacetate (CFD; Molecular Probes, Plano, TX) and 1.25% dimethyl sulfoxide. Phagocytosis by the amoeba of the heat-killed *Tetrahymena* was induced as previously described for *C. carolinensis* (9), and the phagocytotic amoebae were then washed through three pools (2 ml) each of culture medium. The amoeba membrane is impermeable to CFD so that, unlike the situation in many mammalian cells, CFD does not readily cross the membrane to be hydrolyzed and trapped (see Results below). Similarly, when CFD is physically trapped in a phagosome it does not diffuse across the phagosomal membrane.

The net photon counts excited at wavelengths 489 and 452 nm, and the fluorescence ratio of these counts were recorded from the phagosome through a × 16 objective (Zeiss × 16, NA = 0.35) and the microspectrofluorimeter operated in its normal mode (see above). An increase in the net photon counts excited at 452 nm occurred when CFD included in the phagosomes was hydrolyzed to highly fluorescent carboxyfluorescein; a change in the fluorescence ratio (EX₄₈₉/EX₄₅₂) occurred when the pH changed within the phagosomes. As we show below in the Results, CFD was hydrolyzed to carboxyfluorescein by esterases delivered into the phagosome by phagosome-lysosome fusion, and the increase in net fluorescence counts excited at 452 nm was therefore an unambiguous indicator of this event. The presence of a small amount of fluorescence at the beginning of the experiment was due to minimal hydrolysis of CFD during preparation.

Measurements of net photon counts were also recorded on the microspectrofluorimeter from heat-killed *Tetrahymena* and from homogenates of living and heat-killed *Tetrahymena* enclosed within 50-μm thick, rectangular microcapillaries. Living and heat-killed *Tetrahymena* were homogenized by sonification with four 50-W pulses of 10 s each (Ultrasonics with Microtip, model

W185, Plainview, NY). Homogenates were stored on ice until used. Cell counts were made with a standard microscope hemocytometer.

RESULTS

Pinocytosis and Subsequent Intracellular Location of Fluorescein-labeled Ribonuclease

FTC-RNase was ingested by *A. proteus* and *C. carolinensis* at pH 7.0 by adsorptive pinocytosis. The positively charged RNase bound to the highly acidic glycocalyx of amoebae, inhibited normal locomotion, and rapidly initiated the formation of numerous pinocytotic channels leading from the cell surface into the cytoplasm (Fig. 2). Vesicles containing FTC-RNase pinched off from the intracellular bases of these channels. Pinocytotic channels disappeared and normal locomotion resumed when amoebae were washed free of FTC-RNase in culture medium, pH 10.7 (see Materials and Methods). Pinocytotic vesicles containing FTC-RNase were ran-

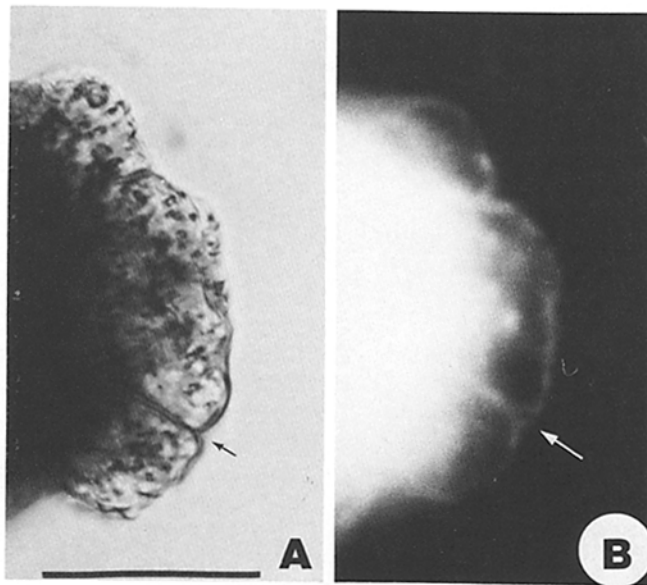


FIGURE 2 Nomarski (A) and fluorescence (B) images of a pinocytotic channel (arrow) of *C. carolinensis* induced in 0.1 mg/ml rhodamine-RNase. Fluorescence images in this and the remaining figures were recorded with an RCA TC1030/H image intensifier as described elsewhere (29). Bar, 100 μ m. \times 300.

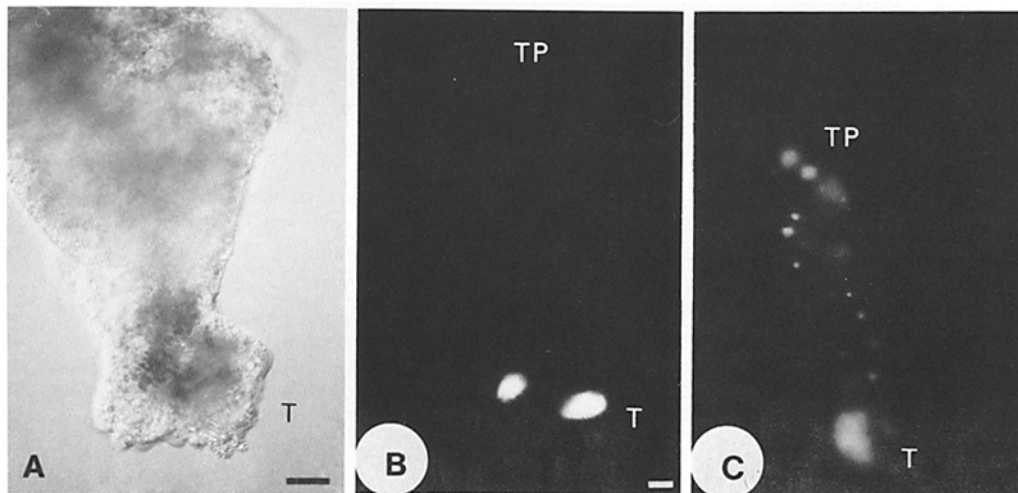


FIGURE 3 Nomarski (A) and fluorescence (B and C) images of *C. carolinensis* photographed 30 min (A and B) and 80 min (C) after initial pinocytosis of 0.1 mg/ml rhodamine-RNase. At 30 min, the majority of the pinosomes are sequestered in the tail (T) of this locomoting cell, but at 80 min they are distributed over the length of the cell. TP, tip of locomoting cell. Bar, 100 μ m. (A) \times 50; (B and C) \times 25.

domly distributed in the cytoplasm of *A. proteus* as soon as it was possible to make observations after washing. By contrast, pinosomes of *C. carolinensis* were concentrated in the tail ectoplasm immediately after initial pinocytosis, where they did not flow with the endoplasm of the locomoting cell (Fig. 3). With time (30–60 min), however, these pinosomes were released from the tail ectoplasm and entered streaming endoplasm with the result that, at 1–2 h after initial pinocytosis, vesicles were evenly distributed throughout the whole cell. At no time in either species was FTC-RNase observed as diffuse cytoplasmic fluorescence.

Standard Curves

The ratio of fluorescence emission excited at 489 nm and 452 nm from FTC-labeled proteins is highly sensitive to pH between 5 to 7 (8, 23). To duplicate as closely as possible in our standard curve the conditions for measuring such ratios from the endosomes of living cells, we collapsed the gradient of pH across endosomes of amoebae by immersing them in a series of weak acid-base buffers (100 mM NH_4OAc , 10 mM Tris-maleate), ranging from pH 5.0 to pH 7.5, and allowed the measured ratio to reach a new and stable value (usually 20–30 min after immersion). The resulting curve of fluorescence intensity ratios versus pH for FTC-RNase in pinosomes is given in Fig. 4. A critical evaluation of this method for calibration using amoebae may be found in Heiple and Taylor (10).

Surface-bound FTC-RNase

Binding of the FTC-RNase to the surface glycocalyx of amoebae reduced its fluorescence intensity ratio on average by a factor of 2.0 ratio U from that measured by the protein in culture medium pH 7.0 (Table I). If this change on binding reflects a pure pH measurement by the FTC-RNase, then the microenvironment at the cell surface of *A. proteus* is maintained as ~ 0.9 pH U lower than culture medium, or, in the present case, at pH ~ 6.1 . Table I shows that chilling cells to 4°C raised the ratio of surface-bound FTC-RNase by as much as 1.03 ratio U, or ~ 0.5 pH U. It did not, however, restore surface pH to 7.0. Ratios measured from surface-bound FTC-RNase varied considerably from one amoeba culture to another but were always lower than that measured from FTC-RNase in culture medium and lower than ratios measured at

pH 7.0 in the in situ calibration. When cells with surface-bound FTC-RNase were rinsed into culture medium pH 6.0, the ratio dropped by 1.3 U (Table I). Thus, surface-bound FTC-RNase is sensitive to changes in the external pH of the medium. If cells with surface-bound FTC-RNase were rinsed in culture medium at pH 10.7 after measurements, all but 0.7% of the fluorescence emitted under 489-nm excitation was removed from the cell. Therefore, the above measurements reflect the ratio of surface-bound probe, not of probe pinocytosed by the cells. Binding of FTC-RNase to the glycocalyx of *C. carolinensis* also reduced the fluorescence intensity ratio (Table I), indicating that this cell too maintains an acidic pH at its surface.

Kinetics of pH Changes within Pinosomes

Pinosomes containing FTC-RNase acidified rapidly in *A. proteus*. In the record of Fig. 5, for example, the pH within the pinosomes of a single cell is less than that at the cell surface as soon as measurement was possible, 5 min after

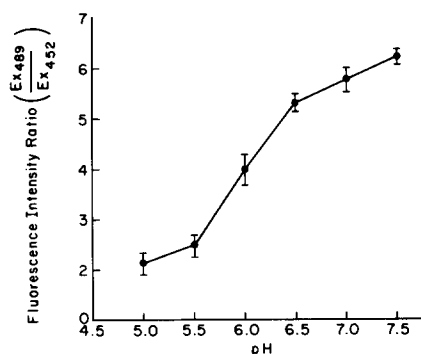


FIGURE 4 Standard curve of fluorescence intensity ratio (Ex₄₈₉/Ex₄₅₂) versus pH for FTC-RNase within pinosomes (●). *A. proteus* that had pinocytosed FTC-RNase were washed three times in 100 nM NH₄OAc, 10 mM Tris-maleate buffer, pH 5.0 to 7.5, mounted in a microscope chamber containing the buffer, and the fluorescence ratios measured from the pinosomes until it was fixed at a new stable value, usually <30 min after initial immersion in buffer. Ratios could not be measured from pinosomes of cells immersed in buffers below pH 5.0 or above pH 7.5, since leakage of FTC-RNase from pinosomes was evident under such conditions. Each point represents the mean of measurements from 3 to 12 cells; the bar represents the standard deviation of the mean.

TABLE I

Effect of Binding to Cell Surface on Fluorescence Intensity Ratio of FTC-RNase

FTC-RNase	\bar{x} Ratio (No. of Cells Measured)	~pH
In culture medium: 25°C, pH 7.0	6.3*	7.0
On cell surface:		
<i>A. proteus</i> , 25°C, pH 7.0	4.3 (6)	6.1 [‡]
<i>A. proteus</i> , 25°C, pH 6.0	3.0 (3)	5.7
<i>A. proteus</i> , 4°C, pH 7.0	5.4 (4) [§]	6.6
<i>C. carolinensis</i> , 25°C, pH 7.0	4.8 (2)	6.3
<i>C. carolinensis</i> , 25°C, pH 6.0	2.8	5.6

* In capillary tube.

[‡] Calibrated from standard curve of Fig. 4.

[§] Cells were preincubated for 30 min at 4°C, exposed to FTC-RNase at 4°C, washed in culture medium pH 7.0 at 4°C, and then transferred to culture medium in an unchilled chamber, and measured immediately.

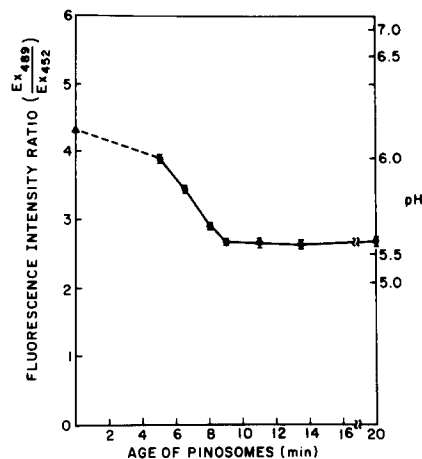


FIGURE 5 Early time course for pH changes within pinosomes of a single *A. proteus* (●). This cell pinocytosed for 2 min before it was washed free of surface bound FTC-RNase with culture medium, pH 10.7. The ratio measured at the surface of *A. proteus* (▲) is also provided for comparison. The bars in this and the following curves represent the standard deviation of the mean of 20 measurements made in a 3-s interval from single cells.

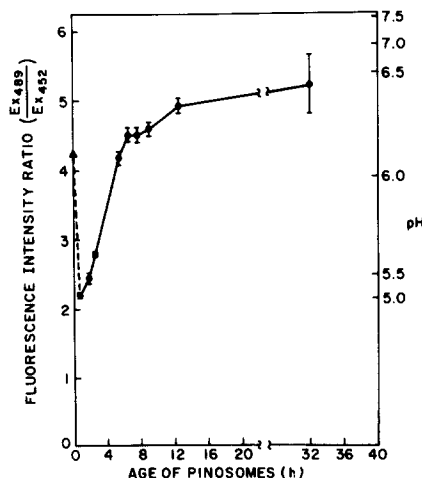


FIGURE 6 Long term time course for changes in the pinosomal pH of a single *A. proteus* (●). This cell pinocytosed 15 min before it was washed. The ratio measured from FTC-RNase bound to the surface of *A. proteus* is also indicated (▲).

initial endocytosis, and the pinosomal pH drops steadily in this cell to a minimum value of pH 5.6 within 8.5 min. Indeed, at 10 min after an initial 2 min of pinocytosis, the contents of pinosomes in 80% of the cells measured was not greater than pH 5.6. After this initial acidification, the pH of pinosomal contents rose steadily during the following 8–10 h reaching pH ~6.4, above which it was maintained for up to 24 h (Fig. 6). *A. proteus* was capable of acidifying its pinosomes in culture medium, pH 10.7 (data not shown). Its pinosomal contents were, therefore, well protected from the high pH of the external environment during washing.

Pinosomal contents of *C. carolinensis* also acidified after initial pinocytosis, but interpretation of the data is complicated by the initially nonrandom distribution of pinosomes in this cell (see above). The pH within pinosomes of whole *C. carolinensis* dropped to ~5.1 at 1–2 h after initial pinocytosis (Fig. 7). But a pinosome's pH in *C. carolinensis* was not simply a function of its age; its cytoplasmic location was also

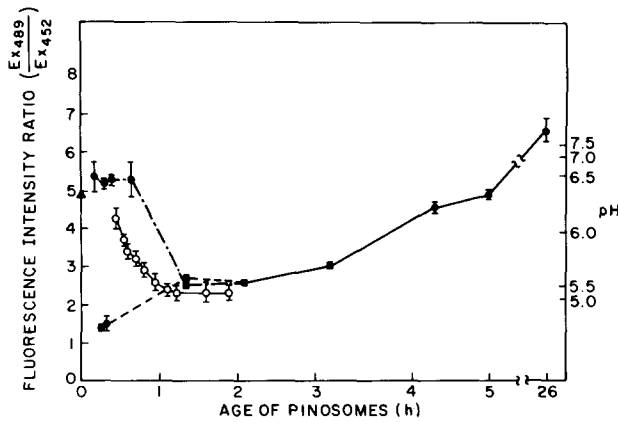


FIGURE 7 Time course for pH changes in pinosomes of *C. carolinensis*. Two kinds of measurement of pinosomal pH are shown: a whole-cell measurement (○) using an × 10 objective, and measurements of selected regions, occupying one-fourth of the whole cell's length, using an × 25 objective. Those regions of the amoebae measured with the × 25 objective are: locomoting tip (●—●), tail (●—●), and mid-region (●—●). Records from cellular tip, tail, and mid-region are recorded from the same amoeba. For clarity, only values of pinosomal pH in cell mid-region are reported after 1.5 h: the ratios measured from tips and tails after 1.5 h were equivalent to one another and to those measured in the mid-region. The record of whole-cell pinosomal pH was made on a separate amoeba. Both cells pinocytosed for 20 min in 0.1 mg/ml FTC-RNase before the wash in culture medium, pH 10.7. The ratio measured by FTC-RNase bound to the surface of *C. carolinensis* is also indicated (▲).

important during the first 50–60 min after initial pinocytosis. The pH of pinosomes initially concentrated in the tail of *C. carolinensis* (see above) was ~6.5, a value maintained for up to 45 min in some cells (Fig. 7). By contrast, pinosomes streaming within the tip endoplasm of *C. carolinensis* were acidic, pH <5.0, as soon as measurement was possible (15 min) (Fig. 7). With time (1.2–1.5 h), pinosomal pH in tips and tails became indistinguishable at pH ~5.1, and pinosomes were no longer concentrated in the tail ectoplasm. The pH within pinosomes from all parts of the cell then rose continually during the next 24 h, reaching slightly alkaline values above pH 7.5 (Fig. 7).

Changes of pH in the culture medium (from pH 7.0 to pH 6.0 or to 9.0) were without effect on the ratio measured from 20–30-min-old tail pinosomes of *C. carolinensis*. Furthermore, washing in culture medium pH 10.7 removed all but 0.5 to 1.0% of surface-bound FTC-RNase from *C. carolinensis*. We conclude that FTC-RNase at the tail of *Chaos* is truly enclosed within pinosomes, where it is inaccessible to culture medium.

Kinetics of pH Changes within Phagosomes

Phagosomal contents, like pinosomal, acidified rapidly in *A. proteus*. Typically, phagosomal pH dropped steadily to pH ~5.4 within 5–10 min of initial phagocytosis, a value 1.6 pH U below the initial pH of the culture medium and FTC-ovalbumin ingested along with the living *Tetrahymena* (Fig. 8).

Lysosomal Fusion with Phagosomes

At various intervals after phagocytosis of living *Tetrahymena*, *A. Proteus* were fixed and their lysosomes (1° and 2°)

were stained by a modification of Gomori's method (3). A black reaction product (Fig. 9) was evident by light microscopy within 20% of the phagosomes at 5 min after initial phagocytosis, and within 40% at 10 min (Fig. 10). By comparison, 70% of the phagosomes had reached a near minimum value of <pH 5.6 at 5 min after initial phagocytosis, and acidification was complete in 90% at 10 min (Fig. 10). 100% of the phagosomes had undergone a measurable acidification at 5 min after initial phagocytosis. Counts from a much smaller sample of phagosomes, recorded in electron micrographs of thin sections, gave slightly higher values (than light microscopy) for the presence of acid phosphatase in phagosomes but confirm that more phagosomes are maximally acid at 5 and 10 min than have detectable deposits of black reaction product. Electron microscopy revealed, in addition, that at 5 and 10 min, when lysosomal fusion is proceeding most rapidly, lysosomes become localized around the phagosome (Fig. 11).

A direct temporal comparison of acidification with the appearance of lysosomal enzyme activity requires that these phenomena are measured simultaneously within a living cell. This was possible with a microfluorimetric assay for lysosomal enzyme activity. It has the following basis: esterase hydrolysis of nonfluorescent CFD yields highly fluorescent carboxyfluorescein (25), and lysosomes characteristically contain such esterases (28). Therefore, upon delivery of lysosomal esterases into a phagosome containing CFD, CFD is hydrolyzed to carboxyfluorescein. This in turn gives rise to an increase in the fluorescence intensity of the phagosome, which can readily be measured with the microspectrofluorimeter. We assume that the esterase activity is optimal at the time of lysosomal fusion.

Living *Tetrahymena* were not suitable particles for phagocytosis in this microfluorimetric assay because of their permeability to CFA and their cytoplasmic esterases. Heat-killing of *Tetrahymena* inactivated their esterase activity, as is shown in Fig. 12 (inset) for whole heat-killed *Tetrahymena* and for homogenized extracts of the heat-killed cells. We therefore induced phagocytosis by amoebae of heat-killed *Tetrahymena* suspended in CFD. Fig. 12 shows how the fluorescence inten-

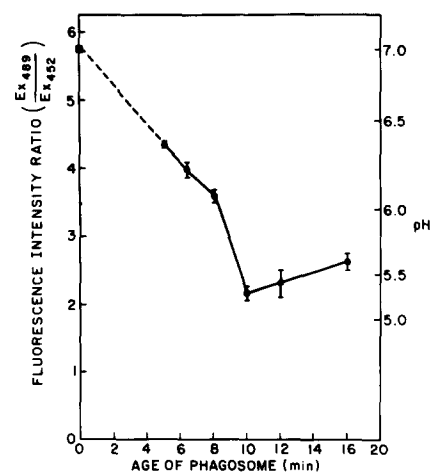


FIGURE 8 Time course for pH changes within a single phagosome of *A. proteus* (●). The indicated values for pH are derived from an in vitro calibration: fluorescence intensity ratios were measured from FTC-ovalbumin (0.1 mg/ml) in 100 mM NH₄OAc, 10 mM Tris-maleate buffer, ranging in pH from 5.0 to 7.5. The ratio measured by FTC-ovalbumin in culture medium pH 7.0 is also given (■).

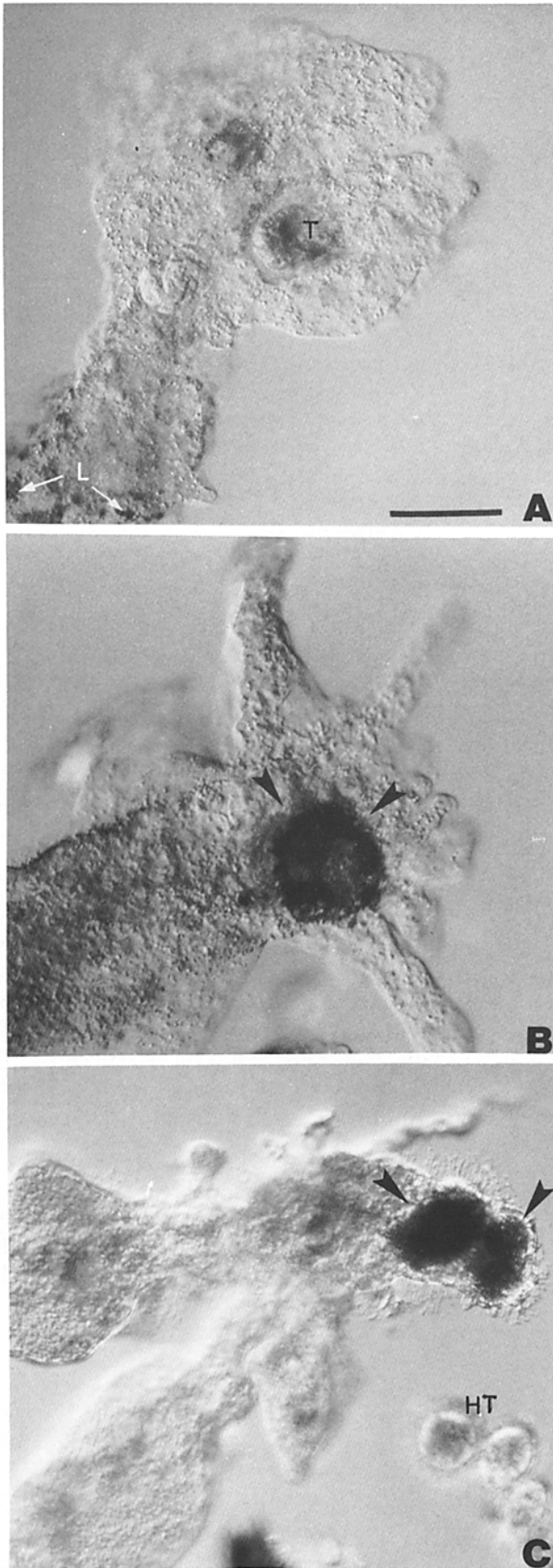


FIGURE 9 Nomarski images of *A. proteus* stained for the lysosomal enzymes: acid phosphatase at 5 min (A), and 20 min (B); and esterases at 10 min (C) after initial phagocytosis of living *Tetrahymena*. The 5-min-old phagosome in A did not stain for the acid

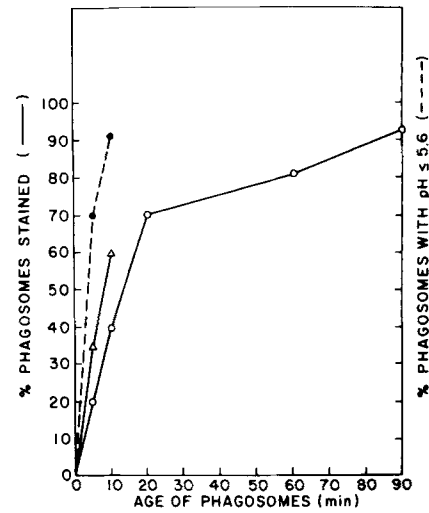
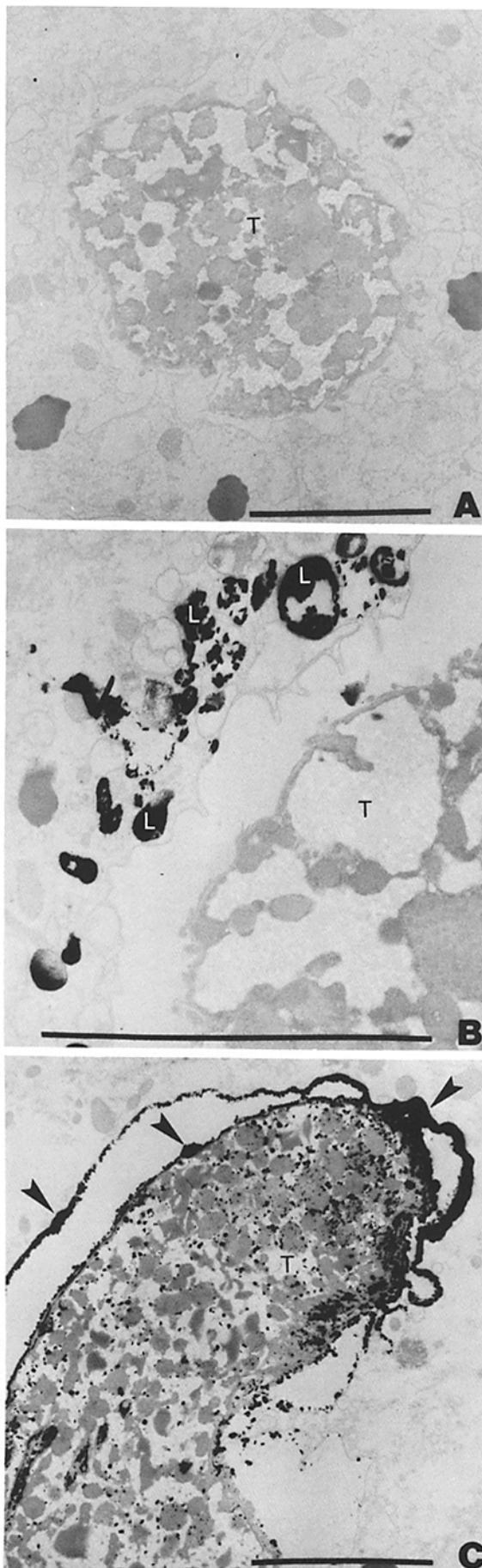


FIGURE 10 Time course for the appearance of lysosomal enzymes in the phagosomes of *A. proteus* as visualized by light microscopy (O) or electron microscopy (Δ), as well as for acidification of phagosomes to pH < 5.6 (\bullet). Each time point for phagosomal staining visualized by light microscopy represents an average of two experiments in each of which 30 cells were scored for the presence or absence of stain in the phagosomes. The 5- and 10-min intervals for electron microscopy represent, respectively, counts of 16 and 15 phagosomes, all amoebae of which were processed in the same experiment. Each time point for acidification represents a mean for measurement of the fluorescence intensity ratio of 12 cells (phagosomes).

sity and fluorescence intensity ratio (measured simultaneously) emitted by the phagosomes of two out of dozens of such amoebae varied as a function of phagosomal age. Fluorescence intensity and fluorescence intensity ratio from the phagosome of amoeba "A" do not change significantly during the initial 3–7 min of measurement; then, at 8 min after phagocytosis, the fluorescence intensity begins to increase steadily for the next 20 min. We interpret this steady increase in fluorescence intensity from phagosomes as an indication of hydrolysis of CFD by lysosomal esterases of the amoebae, and the initiation of the increase in fluorescence intensity as marking the initiation of phagosome-lysosome fusion. The near constant fluorescence intensity often measured in 3–7-min-old phagosomes such as that of amoeba A is interpreted as an indication of phagosomes not yet fused with lysosomes. An increase in fluorescence intensity, or the initiation of lysosomal fusion, was recorded in phagosomes only 3-min-old, and was delayed by no longer than 8 min after initial phagocytosis. Increases of fluorescence intensity were recorded in 29% (7 measured) of 5-min-old phagosomes and in 100% (14 measured) of 10-min-old phagosomes.

The fluorescence intensity ratio emitted by phagosomes was measured simultaneously with fluorescence intensity. Since the fluorescence intensity ratio of carboxyfluorescein is a sensitive indicator of pH (25, 31, and our observations), changes of pH could also be recorded from phagosomes containing CFD and heat-killed *Tetrahymena*. The record of

phosphatase; the 20-min-old phagosome of B did stain. The 10-min-old phagosomes of C were heavily stained blue for esterases, whereas heat-killed *Tetrahymena* (HT) processed along with this amoeba showed no such staining. T, living *Tetrahymena*; L, lysosomes; arrowheads point to deposits of stain within phagosomes. Bar, 100 μ m. \times 150.



amoeba *A* in Fig. 12 shows that, in this particular cell, near complete acidification of the phagosome was achieved before phagosomal fusion was evident as an increase in fluorescence intensity. However, records such as that of amoeba *B* of Fig. 12 indicate that considerable acidification continued in other cells after lysosomal fusion was evident. These two processes, acidification and lysosomal fusion, are therefore closely coupled in time.

The increase in fluorescence intensity measured by the microfluorimetric assay was produced by phago-lysosomes of the amoebae, not by their cytoplasm and its associated esterases. Amoebae immersed in CFD for 5 min but not allowed to phagocytose did not show any increase in fluorescence intensity (Fig. 12). Furthermore, the fluorescence ratios measured from cells with phagosomes were always <3.0 at 10 min after phagocytosis. This would be expected of carboxyfluorescein trapped in acidic phagosomes but not of carboxyfluorescein trapped in cytoplasm whose pH is 6.85 (8). Lastly, the fluorescence of amoebae viewed through the microscope was confined initially to the phagosome containing the heat-killed *Tetrahymena*. The plasma membrane of *Amoeba proteus* is apparently not permeable to CFD, in contrast to the plasma membrane of many cells.

A conventional cytochemical stain for lysosomal esterases was heavily deposited within 10-min-old phagosomes containing heat-killed *Tetrahymena*, but was absent within heat-killed *Tetrahymena* not phagocytosed but stained along with the phagocytotic amoebae (Fig. 9 c). This is independent evidence to confirm that esterases were delivered into phagosomes by the lysosomes of amoebae, and that such esterases were not present in the heat-killed *Tetrahymena* before phagocytosis.

DISCUSSION

We have recorded rapid temporal variations of pH within endosomes of single cells using an improved microspectrofluorimeter and the fluorescein fluorescence ratio technique. Advantages of the fluorescence ratio technique for measuring endosomal pH have been adequately discussed in general elsewhere (8, 10). Our photo-counting microspectrofluorimeter has enhanced the sensitivity with which fluorescence emission can be quantitatively recorded from single cells. This allowed us to record the pH of a small population of pinosomes (numbering as few as 5–10), well defined in time, with signal to noise ratios >10 , and to measure small changes in the fluorescence intensity of single phagosomes. Furthermore, enhanced sensitivity provided excellent spatial resolution within cells: by measuring through higher power objectives, cells could be “scanned” for variations of pinosomal pH at different locations within the cytoplasm.

Cell Surface pH

We found that RNase, on binding to the cell surface, entered a microenvironment of apparently reduced pH rela-

FIGURE 11 Electron microscope images of 5-min- (A) and 10-min- (B and C) old phagosomes in *A. proteus* stained for the lysosomal enzyme, acid phosphatase. Stain (arrowheads) is not evident in the 5-min-old phagosome (A), but is clearly present in the 10-min-old phagosome of C. In B, lysosomes are concentrated around the perimeter of the 10-min-old phagosome, although no fusion, is evident. Sections were not stained with uranyl acetate or lead citrate. L, lysosomes; T, *Tetrahymena*. Bar, 10 μ m. (A and C) $\times 3,000$; (B) $\times 6,000$.

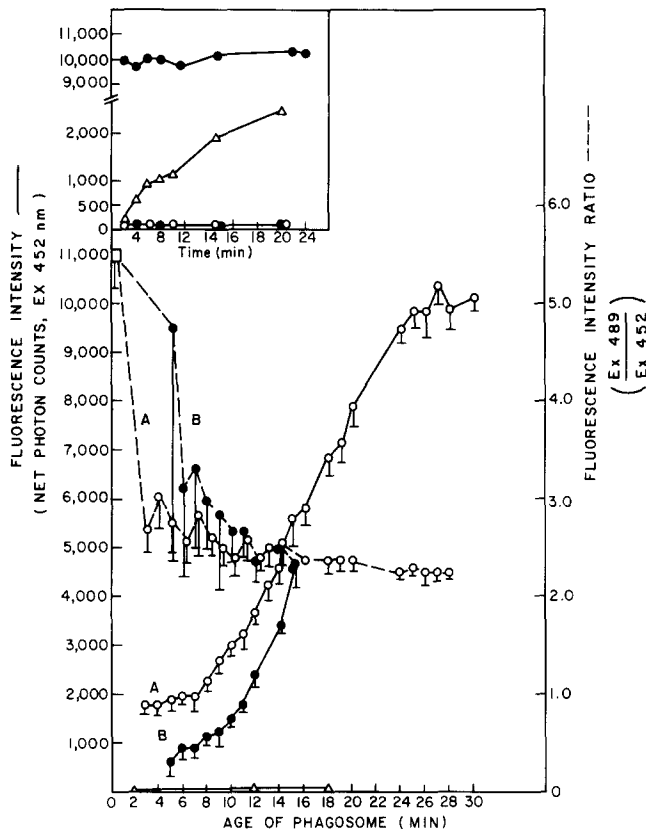


FIGURE 12 Fluorescence intensity and fluorescence intensity ratios recorded over time from phagosomes containing CFD (0.06 mg/ml) and heat-killed *Tetrahymena*. Two amoebae, A (O) and B (●), phagocytosed heat-killed *Tetrahymena* and CFD at time zero. Also shown as a function of time is the fluorescence intensity of an *A. proteus* immersed for 5 min in CFD but not allowed to phagocytose (Δ). Time zero for this control was recorded upon washing of the cell into culture medium not containing CFD. The pH within a phagosome (containing a heat-killed *Tetrahymena* and fluorescent carboxyfluorescein) was clamped in a fourth amoeba at pH 7.0 (using as above 100 mM NH_4OAc , 10 mM Tris-maleate buffer). The fluorescence intensity ratio measured from this phagosome is indicated at time zero (□). Inset: Fluorescence intensity of heat-killed *Tetrahymena* in vitro (upper trace, ●). Heat-killed *Tetrahymena* (preincubated as for phagocytosis experiments in 0.06 mg/ml CFD) were loaded into a rectangular capillary 50 μm in diameter at time zero and the fluorescence intensity measured from a field containing 15 of the cells. Each equivalent of the measured field along the length of the capillary contained one or more *Tetrahymena*. Therefore, diffusion of fluorescent carboxyfluorescein away from the 15 cells measured cannot account for the absence of increasing fluorescence in the record. When a homogenate of living *Tetrahymena* was added to another sample of this suspension of heat-killed *Tetrahymena*, the fluorescence intensity increased over 10-fold in 5 min, showing that unhydrolyzed CFD was present. Also tested for esterase activity (lower traces) were amoeba's culture medium (O) and homogenates of heat-treated (●) and living *Tetrahymena* (Δ). Each of these were mixed at time zero with CFD to give a final concentration of 0.06 mg/ml and then loaded into 50- μm -thick, rectangular capillaries. The concentration of the homogenates were such that the equivalent of 0.75 intact heat-treated and living *Tetrahymena* were measured.

tive to culture medium. Although a reduced pH at the cell surface has long been predicted from what is known of cell surface chemistry (34), the phenomenon, to our knowledge, has not been subject to direct experimental validation. Acidic

pH at the cell surface could result from an energy independent "Gibbs-Donnan type" equilibrium. Thus, the fixed surface charges present on amoeba's glycocalyx (estimated to number 0.5 mEq/l of amoebae) are sufficient, in theory, to lower pH at the glycocalyx by 1.3 pH U from that of a defined culture medium (12). Alternatively, vigorous outward pumping could concentrate protons at the cell surface. Although not studied in amoebae, proton pumps are a common feature of the plasma membrane of other cells, where their apparent function is to reduce cytoplasmic acidity by ejecting protons across the plasma membrane into the culture medium. Whatever may be the mechanism of its maintenance, acidic pH at the cell surface is of interest, because it could affect the characteristics of ligand-receptor binding, the induction of cellular processes in relation to calcium fluxes (30), and because, when the cell surface is incorporated into the endosome, it could provide one means for acidifying endosomal contents.

We cannot presently exclude possible contributions of all physical phenomena other than pH to the reduced ratios we measured from surface bound FTC-RNase, and we cannot, as is possible for endosomal pH, corroborate our surface measurement of pH with those done by a different method. However, Heiple and Taylor (10) showed that the concentration of FTC-ovalbumin, the ionic strength of its aqueous solvent and lowered temperature do not significantly affect the ratio measured at a given pH. Furthermore, we showed that the ratio of FTC-RNase bound to cell surface is sensitive to changes in the pH of the culture medium, and to changes in its temperature, both of which are properties consistent with a pH measurement.

Pinosomal pH

We show that the contents of pinosomes are rapidly and steadily acidified after their initial formation at the cell surface. The pH within pinosomes of whole *A. proteus* dropped to a minimal value of ~ 5.6 within 5–10 min after initial endocytosis; and the pH within pinosomes of the tip endoplasm of *C. carolinensis* dropped below pH 5.0 within 15 min. While, in part, this acidification began on binding of the protein to the cell surface (see above), it continued in the pinosome, whose contents reached a pH at least 1 pH U below that measured on the cell surface. However, acidic pH within pinosomes was temporary in both species: by 24 h after initial pinocytosis the pH within pinosomes of *A. proteus* was ~ 6.4 , and within those of *C. carolinensis* it was ~ 7.5 . In this alkalization, as well as in their rapid initial acidification, pinosomes appear to resemble phagosomes of amoeba (9). No such alkalization of endosomes has been measured from other cells. Because lysosomes fuse with the phagosomes of amoebae in <10 min (see below), the phago-lysosomes or 2° lysosomes of amoebae can assume a pH near or slightly above neutrality. Thus, in amoebae, at least, a subpopulation of 2° lysosomes does not appear to have an acidic pH. By contrast, the 2° lysosomes of macrophages containing FTC-dextran were acidic at 8–36 h after initial pinocytosis (23).

We found that the pH within a pinosome was highly correlated with its location in the cell. Tail pinosomes of *C. carolinensis* were initially pH ~ 6.5 ; those in the cell's tip were less than pH 5.0. The release of pinosomes initially concentrated in the tail of *C. carolinensis* corresponds in time to a decline in pH of the total population of pinosomes, and an acid pH was always recorded in the tip of *C. carolinensis* at 0 to 2 h after pinocytosis. We speculate, therefore, that a

pinosome of pH \sim 6.5 may be trapped by tail ectoplasm until it acidifies, and that acidification could regulate its release into endoplasm.

Phagosomal pH

The pH within phagosomes of *A. proteus* dropped in 10 min or less from 7.0 to a minimal pH of 5.3–5.6. In a qualitative sense, the phagosomes of amoeba initially resemble those of all other cells studied (6, 9, 14, 15, 18), and, in fact, our minimal phagosomal pH is remarkably close to Mast's (18), who, 40 years earlier, provided an estimate of pH \sim 5.6. Despite such qualitative similarities, the rates at which phagosomes acidify vary considerably between cells. The pH within phagosomes of *C. carolinensis*, for example, did not reach a minimum value of \sim 4.9 until 20–30 min after initial phagocytosis (9). Similarly, the pH within pinosomes of whole *A. proteus* dropped considerably faster than the pH within pinosomes of whole *C. carolinensis*. Since in both our study and in Heiple and Taylor's (9) the amoebae phagocytosed one living *Tetrahymena*, differences between the two species in their kinetics of acidification of phagosomes cannot be a function solely of particle type, or of the extent of phagocytosis by the cells. Instead, the rate of endosomal acidification is probably a property peculiar to cell species or type. Such differences in rates of acidification between cells may reflect their use of fundamentally distinct mechanisms of endosomal acidification, or use of a common mechanism that precedes at various rates.

Initiation of Lysosomal Fusion with Phagosomes

It is obviously no longer the phenomenon but the mechanism of phagosomal acidification, and its role in the phagosome, which remain to be determined. Endosomes could acidify by two general mechanisms: (a) lysosomes could fuse with endosomes, mixing their acidic contents (23) with those of the endosome and/or delivering into endosomal membrane those proton pumps now known to reside in membranes of 2° lysosomes (22); or (b) endosomes could acidify by means intrinsic to the plasma membrane, which originally encloses the molecule or particle at the cell surface. Our preliminary evidence, concerning the acidifying potential of cell surface, is consistent with the second mechanism. Further evidence compatible with the second mechanism comes from our time course for the appearance of lysosomal enzyme activity in phagosomes. As judged by cytochemical staining, lysosomal fusion with phagosomes was rapid, but was slightly slower than acidification. However, cytochemical staining is at best a semi-quantitative assay for lysosomal fusion. Neither light microscopy of whole phagosomes, nor electron microscopy of thin sections through phagosomes, provide unambiguous criteria by which to judge that no lysosomes had fused with the phagosome in question. In both cases stain can be present in the phagosome but missed by the microscopist. In addition, using cytochemical techniques, comparisons of the rates of lysosomal fusion with rates of acidification cannot be made within a single cell, but require averaging of the data from populations of cells.

Our microspectrofluorimetric assay for lysosomal esterase activity minimized these problems. We used it to show that near complete acidification of the phagosome was achieved in some cells before phagosomal fusion with lysosomes was detectable (defined operationally here as the appearance of

lysosomal esterase activity). But in measurements from other cells the microfluorimetric assay indicates that acidification continued after lysosomal fusion was initiated. We conclude that acidification of phagosomes by *A. proteus* is initiated but not necessarily completed prior to phagosome-lysosome fusion, and we suggest that the two events are closely linked in time. Evidence from the cytochemical and fluorescence assays are both not consistent with the concept that acidification of a phagosome depends on its fusion with lysosomes.

The microspectrofluorimetric assay can make simultaneous and unambiguous records of acidification, and of initiation of phagosome-lysosome fusion, within the phagosomes of single, living cells. Initiation of fusion of 1° and/or 2° lysosomes with phagosomes is recorded as a highly reproducible and quantitative rise in fluorescence intensity. Nevertheless, as we have used it, the technique is limited in its application. Because *Amoeba proteus* is impermeable to CFD, its cytoplasm does not become fluorescent during a 5-min immersion in the CFD. This is not the case for many cells, including most mammalian phagocytes. We have, however, used carbodiimide to couple CFD to latex beads possessing surface amino groups (unpublished observations). Such CFD-latex could be presented for phagocytosis to other cell types in the absence of CFD free in the culture medium, and phagosome-lysosome fusion could then be followed microphotometrically as here for *A. proteus*. Alternative fluorogenic substrates are now being developed in Dr. Alan Waggoner's laboratory (Carnegie-Mellon University) which will, we hope, enable us to measure lysosomal fusion with pinosomes.

The suggestion that endosomes are acidified prior to endosome-lysosome fusion has been made before. Geisow et al. (6) showed that agents believed to perturb fusion of 2° and/or acidic lysosomes with phagosomes (5) had little effect on acidification of the phagosomes of macrophages. Tycko and Maxfield (32) presented light and electron micrographs showing that little Gomori stain was present in pinosomes of fibroblasts at 5–20 min after interiorization of α_2 -macroglobulin at the cell surface. These 5 to 20 min old pinosomes had an average pH measured from a population of fibroblasts of \sim 5.0. Unfortunately, since quantitative data for Gomori staining was not presented for a large sampling of individual fibroblasts, it is not possible to determine whether those micrographs presented were representative of the pattern of lysosomal staining and/or of a cell with an average pinosomal pH of 5.0. It was our experience with amoebae that both the staining patterns and the kinetics of acidification of phagosomes varied considerably from one cell to another. Flow cytometric analysis has indicated that pinosomes containing insulin fused with lysosomes of 3T3 cells 30 min after initial endocytosis (21).

Studies by Bainton and co-workers (2, 15) on the degranulation of leukocytes stand in noteworthy contrast to those just reviewed above. Bainton (2) visualized stain for alkaline phosphatase, a marker enzyme for the leukocyte's "specific" granules, within phagosomes as soon as 30 s after the initiation of phagocytosis, whereas the pH of these phagosomes did not drop to \sim 6.5 until 3 min after phagocytosis and did not drop to a minimum value of pH \sim 4.0 until 7–15 min after phagocytosis (15). Whether the counterpart of these "specific" granules exists in other cells has not been determined.

Conceivably, acidification of the phagosome could regulate its subsequent fusion with lysosomes, which appear by electron microscopy to cluster around the phagosome prior to

their fusion with it (Fig. 11 *b*). Semlicki Forest virus fuses with host cell membranes when the pH of its microenvironment is reduced to <6 (11, 18). But this system cannot serve as an adequate model for endosomes, since, if pH changes occurring within the endosome are supposed to regulate fusion with lysosomes, they must somewhat affect the interaction of the outer surface of the endosomal membrane with the outer surface of the lysosomal membrane. Transmembrane signaling is often accomplished by rapid changes in ion gradients. A proton gradient is clearly established across the endosomal membranes of amoebae, since the pH of the cytoplasm in these cells is ~6.8 (8). The close temporal relationship which we show exists between initial endosomal acidification and lysosomal fusion with endosomes is, therefore, compatible with a role for endosomal acidification, perhaps as a trans-membrane signal, in regulating endosome-lysosome formation. The near neutral pH of older phagosomes and pinosomes could, conversely, act as a signal preventing further delivery of superfluous lysosomal enzymes into the endosome. These possible roles for changing endosomal pH in regulating lysosomal fusion are not incompatible with the previous suggestion that such pH changes regulate the activity of the numerous pH-sensitive enzymes of the digestive endosomal vacuole.

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Note Added in Proof:—Galloway et al. (*Proc. Natl. Acad. Sci. USA*, 1983, 80:3334–3338) show that 1–5-min-old pinosomes of macrophages and fibroblasts can be separated from lysosomes on density gradients, and that such pinosomes are capable of self-acidification. They suggest that these 1–5-min-old pinosomes represent an acidic, prelysosomal compartment. This compartment therefore resembles that of the 3–5-min-old acidic, prelysosomal phagosomes of amoeba studied in the present paper.

Murphy et al. (personal communication) used fluorescence flow cytometry to show that endosomes of single, living cells containing insulin and α_2 -macroglobulin are both acidic as soon as 10 min after initial pinocytosis.

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