Temporal Changes of Lysosome and Phagosome pH during Phagolysosome Formation in Macrophages: Studies by Fluorescence Spectroscopy

MICHAEL J. GEISOW, PHILIP D'ARCY HART, and M. ROBERT YOUNG
National Institute for Medical Research, London, England

ABSTRACT Intravacuolar pH was measured within the lysosomes and newly formed phagosomes in cultured mouse peritoneal macrophages. The kinetics of pH change in both vacuolar systems was quantitatively determined within a large cell population by fluorescence spectroscopy. Additionally, pH changes within individual phagosomes were followed semiquantitatively using indicator dyes.

Two novel findings were made. Firstly, the pH in new phagosomes was transiently driven alkaline (higher than physiological) even when the external medium was buffered at pH 6.5. Secondly, perturbations of phagosome-lysosome fusion had little effect upon phagosomal pH changes, even though the compounds used markedly altered the pH of the lysosomes in resting and phagocytosing cells.

A pH-sensitive fluorescent probe has been employed to measure pH and "microviscosity" within living macrophage lysosomes and phagosomes during the fusion of these organelles (P-LF). Fluorescein was specifically introduced to phagosomes or lysosomes by conjugation with the surface of yeasts or dextran, respectively. The resting pH of lysosomes in mouse peritoneal macrophages and baby hamster kidney (BHK21) cells in culture was 4.8 ± 0.1, in close agreement with the value reported by Ohkuma and Poole (1) for macrophage lysosomes. Phagocytosis of yeasts provoked a rapid rise in lysosomal pH, and the subsequent P-LF was characterized by a chronically raised pH (5.4 ± 0.2) recorded by the fluorescent probe. The initially high fluorescence polarization of the probe (0.33) gradually decreased during P-LF, reflecting its transfer within the cells to a less restricted environment (i.e., the phagosomes). Conversely, fluorescein conjugated to yeasts experienced a rapid fall in pH after entering the cells, finally monitoring the same average pH as the lysosomal probe (5.4 ± 0.4). When phagocytosis of fluorescent yeasts was synchronized by prebinding the yeasts at 4°C, there was a transient rise in fluorescent emission that was maximal 2 min after the initiation of phagocytosis. The maximum recorded pH was 7.75, higher than that in the extracellular medium. Thereafter the pH of the phagocytic vacuoles decreased as before. The kinetics of this pH change are reminiscent of those of the respiratory burst of neutrophilic leukocytes (2).

Weak bases produced an immediate increase in lysosomal pH, but had little effect upon the pH of newly formed phagosomes. Polyanions that inhibit P-LF (3) did not alter resting lysosomal pH or the mobility of the lysosomal fluorescent probe. They also had no effect upon the early pH changes of the phagosomes, but prevented the sustained increase of lysosomal pH during phagocytosis. It is suggested that the early changes in the phagosomal pH are unrelated to the fusion of lysosomes that can be labeled with weak bases (acidic lysosomes) or dextran (secondary lysosomes). The ability to regulate phagosome pH must either be intrinsic to the unfused phagosome or be conferred by fusion of granules transparent to the normal labeling methods.

After the uptake of particles into leukocytes by phagocytosis, the resultant phagocytic vesicles fuse rapidly with lysosomes and the intravesicular space becomes acid. Jensen and Bainton (4), using indicator dyes absorbed to yeast cells, provided approximate measurements of phagosomal pH and later (5) showed in vitro that neutrophil and monocyte phagosomes became acidified to pH 4.5–5.0 within 10 min of ingestion. We have extended these observations with macrophages in monolayer culture on glass by selectively and separately introducing fluorescent probes into lysosomal and phagosomal compartments. Secondary lysosomes were labeled with a metabolically stable fluorescein conjugate and fluorescein was introduced into phagosomes by chemical conjugation of the probe with

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the surface of live yeast cells. Using a fluorescence spectrophotometer, we made quantitative observations of intracellular pH within these organelles during the process of P-LF.

We have recently reported (3) the suppression of P-LF in macrophages by a semisynthetic polyanion, chlorite-oxidized amylose (COAM). In the present study this compound has been used to observe the consequences of inhibition of P-LF upon the pH of lysosomes and phagosomes. The term phagosome in this article should be taken to imply "phagolysosome" unless stated otherwise.

MATERIALS AND METHODS

Fluorescent Conjugates

Fluorescent dextran with an average molecular weight of 40,000 (FD40) made by reaction of fluorescein isothiocyanate (FITC) with dextran was obtained from Sigma Chemical Co, St. Louis, Mo. FITC was conjugated with the surface of live *Saccharomyces cerevisiae* after extensive washing of the cells in saline and water. 50 μl of packed cells was suspended in 0.8 ml of 50 mM pyridine-HCL, pH 5.1, at 4°C. To this solution, 50 μl of fluorescein amine isomer 1 (Sigma Chemical Co.), 1 mg/ml in dimethyl sulfoxide (DMSO) was added, followed by 3 mg of 1-ethyl-3-(dimethylaminopropyl)carbodiimide in 50 μl of water. After 4 h of mixing by inversion at 4°C, the cells were washed well in cold 25% DMSO and then saline. Examination of the cells in a fluorescence microscope showed that they were uniformly labeled on their surfaces only. In a few experiments, yeasts were immunofluorescently tagged with an FITC-labeled antiyeast antibody prepared in this Institute. After such labeling, the yeasts remained viable.

Tissue Culture

The cells used were either normal mouse peritoneal macrophages established in culture as monolayers on 3.5 × 1 cm cover slips (3) or BHK21 cells grown for 16 h to near confluency in Dulbecco's H21 medium. Outside culture, cells were handled in Hanks' balanced salt solution (BSS) containing 5 mM glucose. FD40 was routinely introduced into lysosomes by incorporation into the culture medium at a concentration of 2 mg/ml for 24 h (macrophages) or 16 h (fibroblasts). Fluorescent yeasts were taken up into macrophages from solutions containing 5–50 × 10^5 yeasts/ml of BSS. After 5 min, the Leighton tubes containing the tissue culture were washed with BSS to remove unbound yeasts, as described in the Results, ensuring a defined "pulse" of yeast uptake. The amount of FD40 taken up was determined in macrophages by dissolving the monolayer in hot 1% SDS. Corrections were made for adsorbed or entrained fluorescence as previously described (3). After exposure to FD40, BHK cells were removed from the glass cover slip with trypsin and washed by centrifugation before extraction of the fluorescent dextran with phosphotungstic acid/perchloric acid. Fluorescein was measured in a MPF4 fluorescence spectrometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) against standards.

Fluorescence Measurements on Living Cells

Cover slips carrying tissue culture were mounted in Perspex end plates sliding on stainless steel tubes. The latter were used to perfuse the fluorescence cuvette at 37°C. The cover slips were mounted at an angle to the incident beam of an MPF4 fluorescence spectrophotometer so that the cells faced the emission monochromator and direct reflections from the glass were deflected away from the detection system. The instrument was operated in ratio mode with narrow (6 mm) band-pass slits. Comparable detection sensitivity was maintained over the period of the experiments by setting up a consistent response amplitude, using rhodamine B dispersed in plastic as a fluorescent standard. Despite the optimized operation of the instrument to minimize the detection of scattered incident light, corrections for scatter from the monolayers were necessary. Each intensity measurement was corrected first for extracellular fluorescence, determined by removal of the cell monolayer from the cuvette, and then for scattered light. Scattered light corrections at a given wavelength were determined from the ratio of light scattered at the same wavelength from cells containing no FD40, to the total light scattered at a wavelength outside the fluorescein absorption spectrum. This last value was obtained for all monolayers by setting excitation and emission monochromators to 575 mm. Polaroid excitation and emission filters were used in the polarization measurements. The measurements at each orientation of the polarizers were separately corrected for scattered light. Polarization measurements were also obtained from FD40 in glycerol solutions of known viscosity under identical conditions.

Isolation of FD40-containing Lysosomes

Cultured macrophages were allowed to take up FD40 (2 mg/ml) for 16 h. After a 15-min "chase" period in BSS at 37°C, the cells were swollen in 20 mM HEPES-KOH, pH 6.5, at 37°C. The rounded cells were removed from the culture bottle with a silicone rubber blade and homogenized (10 strokes of the tight-fitting pestle of a Dounce-type homogenizer) in the HEPES buffer at 4°C. Isotonicity was restored by addition of sucrose solution. Unbroken cells and nuclei were removed by centrifugation at 850 *g* for 15 min. The supernate was layered onto a gradient of 20–50% wt/wt sucrose overlying a 60% wt/wt sucrose cushion and centrifuged 90 min at 150,000 *g*. 0. 5-ml fractions were collected and examined for fluorescein fluorescence in an MPF4 spectrophotometer. Aliquots were disrupted ultrasonically (Dawe Sonniprobe, type 1130A; Dawe Instruments Ltd., London, U. K.; 3 × 10 s at setting 1). 0.2 ml of each sonicate was added to 0.2 ml of 40 mM sodium acetate, pH 5.0, and 0.1 ml of 6 mM 4-methylumbelliferone phosphate was added. After 90 min, the enzyme reaction was terminated by 0.5 ml of 0.2 M sodium glycinate, pH 10.4. Acid phosphatase activity was determined as phosphate released per hour, from the fluorescence of the released 4-methylumbelliferone (6).

RESULTS

Uptake and Intracellular Location of Fluorescent Dextran

The incorporation of FD40 into the culture medium of macrophages or BHK cells gave rise to a slow accumulation of visible fluorescence within perinuclear cytoplasmic granules (Fig. 1). No fluorescein fluorescence was observed associated with other identifiable cell structures. The uptake of dextran was linear with time and concentration and probably occurs via a nonspecific pinocytic pathway (7, 8). The rate of uptake determined for macrophages (0.4 μg/mg of cell protein per hour) is comparable to the value reported by Ohkuma and Poole (1) (0.25 μg/mg of cell protein per hour). A higher rate (1.25 μg/mg of cell protein per hour) was observed for BHK cells. The polyanion, COAM, was internalized much more rapidly by macrophages (40 μg/mg of cell protein per hour), indicating that this material may enter by adsorptive as well as fluid pinocytosis.

When postnuclear homogenates of macrophages containing FD40-labeled lysosomes were centrifuged on sucrose density gradients, fluorescent fractions were obtained as shown in Fig. 2. The distribution of fluorescence on gradients corresponded with lysosomal acid phosphatase activity with a peak at a sucrose density of 1.18 g/cm^3. This value also corresponds to the buoyant density in sucrose of 1.18 g/cm^3 reported for normal mouse peritoneal macrophages (9). Relatively pure, discrete fluorescent granules were visible in the peak fractions when examined in the fluorescence microscope. It is clear that the concentration of FD40 in macrophage lysosomes under the conditions used for subsequent pH measurements did not significantly perturb their buoyant density. These lysosomes therefore contrast with the "dextranosomes" produced by dextran in rat hepatic cells. Such lysosomes sediment to the bottom of similar density gradients (10).

Observations of Lysosomal pH

The excitation spectrum of fluorescein is very sensitive to pH between 5 and 7. A change of 0.1 pH unit produces a substantial change in the emission characteristics and can be measured with confidence. Acid quenching alters the shape of the spectral envelope, and so we adopted the suggestion of Ohkuma and Poole (1) that the ratio of emission at two wavelengths could be used as a more reliable pH index than absolute intensity. The 490 nm/450 nm emission ratio as a function of pH is reproduced in Fig. 3 for the two probes (FD40 and fluorescent

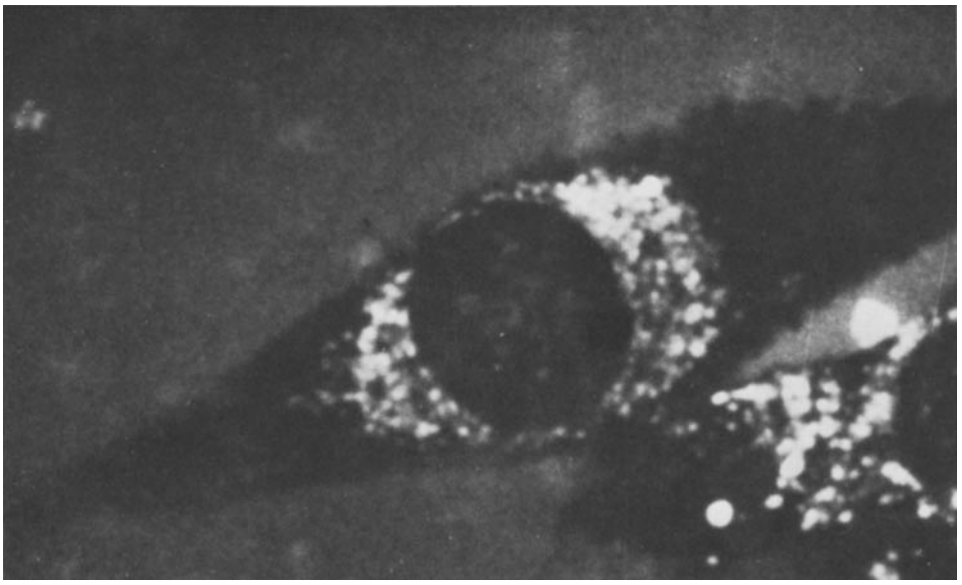

FIGURE 1 The appearance of BHK21 cell lysosomes grown for 16 h in the presence of fluorescent dextran. × 4,280.

yeasts) free in solution. This ratio was insensitive to changes in FD40 concentration and to alteration of the ionic composition of the solution. Macrophage monolayers containing FD40-labeled lysosomes produced the excitation spectrum shown in Fig. 4 *c*.

Representative lysosomal pH was determined as 4.8 ± 0.1 for macrophages and BHK cells freshly removed from culture medium. After prolonged incubation at 37°C in BSS, the pH generally increased, especially in the more sensitive BHK cells. The addition of a weak base such as methylamine or ammonium chloride to the BSS in the fluorescence cuvette initiated a rapid rise in lysosomal pH as apparent from the spectral changes indicated in Fig. 4 *a* and pH kinetics shown in Fig. 5 *b*. Both chloroquine and ammonium chloride produced reversible pH changes in the lysosomes, but hysteresis in the recovery of the original pH was observed upon removal of all weak bases except ammonium chloride from the perfusing medium (Fig. 5). Minor changes in external pH (e. g., produced by different buffering in the BSS) did not affect lysosomal pH, nor did the reduction of the temperature of the BSS to 15°C. The lysosomal pH was steady for at least 1 h during perfusion of the fluorescence cuvette with BSS. At the end of that time, the macrophages were still able to phagocytose yeasts, despite intermittent high-intensity illumination.

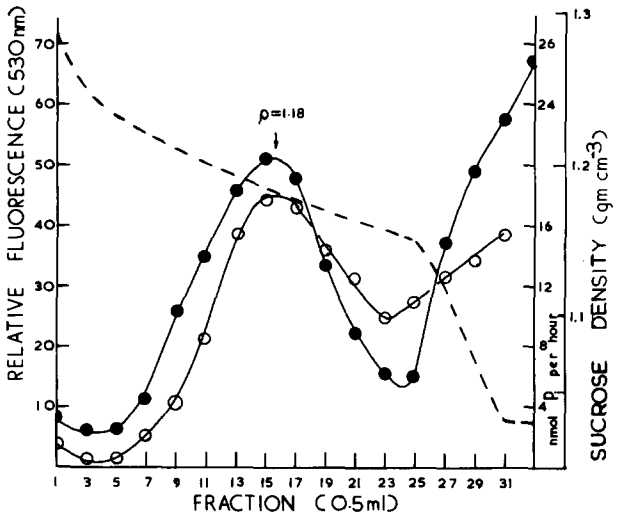

FIGURE 2 Sucrose density gradient fractionation of macrophage lysosomes containing FD40. The gradient runs from 20–50% wt/wt sucrose on a 1-ml cushion of 60% wt/wt sucrose. Alternate fractions were assayed for fluorescein (relative fluorescence) (●) and acid phosphatase (P_i activity (○). Dashed line represents sucrose density.

Polarization of Intralysosomal FD40 Fluorescence

The polarization of fluorescein fluorescence gives information about molecular rotations on a time-scale shorter than 5 ns, the fluorescent lifetime of free FITC. For fluorescein conjugates, the molecular rotation of the chromophore is obviously coupled with that of the accompanying macromolecule. Fluorescence polarization of conjugates will thus depend upon the restrictions of freedom of rotation placed upon the total conjugate by the microviscosity of the immediate environment. Fluorescence polarization of FD40 was measured as a function of the viscosity of buffered glycerol solutions (Table I). Because the polarization changes at both low and maximum viscosity at 37°C were rather small upon conjugation, it was clear that the rotational freedom of fluorescein conjugated to dextran remained high and that the fluorescent lifetime of the excited state was not significantly changed by conjugation. Similar results were obtained from studies of tetramethylrhodamine conjugates.

The polarization of lysosomal FD40 fluorescence was high and constant for a minimum of 1 h ($P = 0.33 \pm 0.2$; $n = 6$). This value was determined at the fluorescence maximum (519 nm) and was reproducible in separate tissue culture batches. A comparable polarization was obtained when FD40 was dissolved in 90% glycerol at 37°C (100 centipoise [cp]). It must be pointed out that this value cannot be taken directly to represent the microviscosity until it has been established that the lifetime of the excited state is unchanged when the probe is within lysosomes. Conjugation alone is unlikely to change the lifetime of fluorescein—early studies have established that conjugation of fluorescein to much more rigid protein structures left its

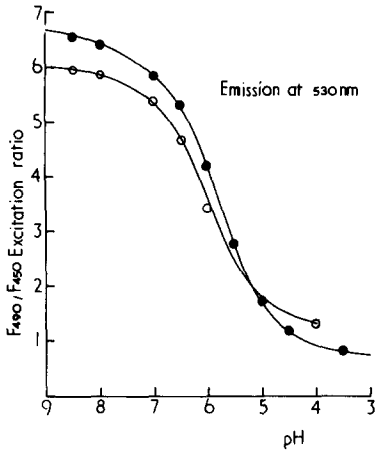

FIGURE 3 Ratio of fluorescent emission from fluorescein conjugates at 490 nm and 450 nm as a function of pH. The curves correspond to FD40 (1 μg/ml) in buffered solution (●) and fluorescein-conjugated yeast (50 μg/ml suspension) (○).

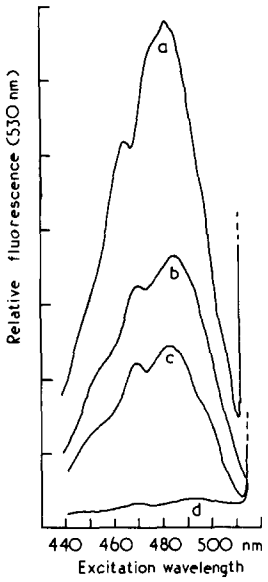

FIGURE 4 Excitation spectra of FD40 within lysosomes in a living monolayer of macrophages. (*d*) Cells not exposed to FD40. (*c*) Cells exposed to FD40, 2 mg/ml for 24 h. (*b*) FD40 free in solution pH 5.0. Addition of 10 mM methylamine to the cells in *c* produced spectrum *a*.

lifetime unchanged and independent of pH between 4.5 and 10 (11).

Effect of Phagocytosis upon Intralysosomal pH

Macrophages with FD40-containing lysosomes were incubated with live yeast cells (3–6 × 10^7/ml) in BSS for 5 min at 37°C. After this yeast pulse, the monolayers were washed in BSS, removed, and observed either in the fluorescence spectrometer or the dark field of a fluorescence microscope. Immediately after the pulse, the majority of yeast cells associated with the monolayer were firmly bound, but few were inside macrophages. During the next 10 min, nearly all the yeasts were internalized. 15 min from the start of the pulse, P-LF became visible to microscopic observation by the appearance of fluorescent rims surrounding the phagocytosed yeasts. This process continued for as long as observations were made (2 h). Spectroscopic observations made during this period indicated that within the first 5 min of exposure to yeasts, the lysosomal pH rose rapidly from pH 4.8 and continued to increase slowly during endocytosis and P-LF, reaching pH 5.4 ± 0.2 after 30 min (Fig. 6).

2 h after ingestion of yeasts, the addition of chloroquine to the BSS perfusing the macrophages still promoted a rapid increase in the FD40 fluorescence. Because a substantial proportion of the lysosomes remained unfused at this time, it was not possible to obtain meaningful pH values from the FD40 that had been transferred to the phagosomes. Observations of FD40 fluorescence polarization were made during this period. 3 h after ingestion of yeasts, the polarization had decreased from 0.33 to 0.23 (n = 3), reflecting an increased mobility of the fraction of the fluorophore transferred to the phagolysosomes by P-LF.

Effect of Polyanions on Lysosomal pH

Macrophages were separately incubated with the P-LF inhibitors poly-D-glutamic acid and COAM as described previously (12). The same cells were then exposed to FD40 (2 mg/ml for 24 h). Lysosomal pH measured fluorimetrically in polyanion-containing cells was always slightly raised (pH 5.0 ± 0.1). The polyanions had no detectable effect upon the polarization of FD40 fluorescence, which remained at 0.33. When ammonium chloride (or another weak base) was added to the fluorescence cuvette, the lysosomal pH rose in the same manner as in the absence of polyanion (Fig. 5 *a*). In both cases there was an immediate reduction of fluorescence polarization to 0.2, which reverted to 0.33 after the ammonium ions were washed out.

Although the presence of polyanions within the macrophage lysosomes was not obvious from alterations in the resting lysosomal pH or microviscosity, differences between the normal and polyanion-containing lysosomes became apparent after the cells had ingested yeasts. Whereas the lysosomal probe recorded an elevated pH (5.4) during normal P-LF, in macrophages pretreated with COAM the probe recorded only a temporary pH increase, after which the pH returned to its original value (pH 5.0 ± 0.1) (Fig. 6).

Observation of Phagosomal pH

The pH of phagosomes was measured by fluorescein amine conjugated to the surface of yeasts (Fig. 7 *a*). Sufficient fluorescent yeasts bound to the macrophage monolayer at a concentration of 5 × 10^5 yeasts/ml to produce an easily measurable fluorescence after a 5-min preincubation at 37°C. For recording of fluorescence, the preincubated cover slips were washed vigorously at 20°C in BSS to remove free or weakly adherent yeasts and mounted in the fluorescence cuvette, which was subject to a continuous perfusion by BSS at 37°C. Fluorescent yeasts were evidently entering the macrophages as the monolayer was transferred to the cuvette, because the pH fell rapidly from 6.8 to below 5.8 within 10 min of the start of the exposure to yeasts. At this time yeasts were still external, because the fluorescent signal responded to minor changes in external pH when the perfusate buffering was altered. The initial rapid drop in pH was followed by a more gradual reduction over the remainder of the period of observation (Fig. 8). During this stage P-LF occurred in parallel studies in which transfer of FD-40 fluorescence from secondary lysosomes to yeast phagosomes was observed by fluorescence microscopy.

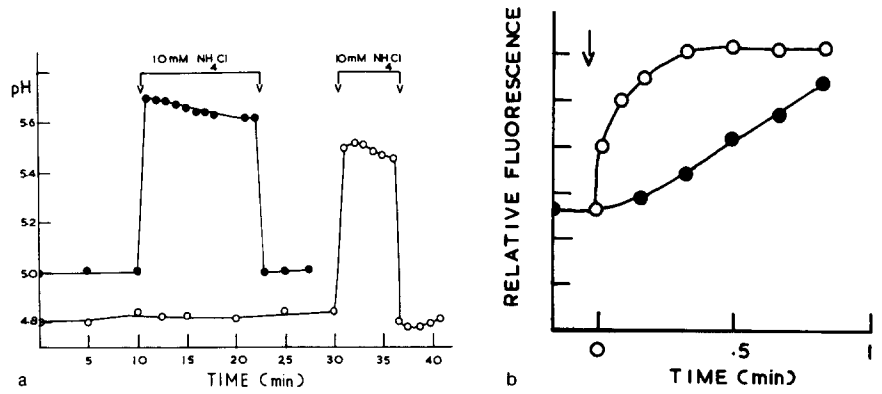

FIGURE 5 (*a*) Lysosomal pH measured by FD40 emission. Lysosomes contained FD40 alone (○) or in addition to COAM (●). At a predetermined time, the monolayer was exposed to 10 mM NH_4Cl BSS by rapid transfer of the cells to a cuvette containing the base. The rate of change of fluorescence is indicated in (*b*) where the base was 10 mM NH_4Cl (○) or 10 mM CH_3NH_2 (●).

The lowest phagosomal pH recorded was pH 5.0; however, the final pH measured in phagosomes was quite variable between different batches of cells.

To reduce the variable uptake inherent in this yeast-pulse technique, cell monolayers were cooled to 4°C and exposed to similar concentrations of yeasts coated with FITC-labeled rabbit antiyeast IgG for 20 min. After extensive washing in cold BSS, the monolayers were placed in the fluorescence cuvette as before. The time taken for the cell layers with adsorbed yeasts to rewarm was probably a matter of seconds. During this time it was anticipated that the fluorescein emission would decrease steeply because of the depression of fluorescence with temperature and the initiation of phagocytosis. Instead, the F_{490}/F_{450} emission ratio increased above that of the external medium, became maximal 1.5–2 min after rewarming, corresponding to a maximum pH 7.75; then by 5 min (the earliest observation time in experiments carried out entirely at 37°C), the pH was decreasing rapidly (Fig. 9). That this apparent rise in pH of early phagocytic vacuoles might be an artifact introduced by the cooling or rapid warmup seems unlikely, because reducing the normal yeast binding time to 2 min at ambient temperature also allowed observation of a transient pH increase. When the 4°C incubation and warmup were carried out in the presence of 5 μM cytochalasin B, phagocytosis was delayed and the pH changes observed in the absence of the drug were similarly reduced and delayed (Fig. 9). All the monolayers examined after fluorescence spectroscopy observations showed no evidence of extracellular yeasts—the fluorescent organisms were always present within the cell periphery and came to focus within the focal depth corresponding to the macrophage. At this stage, the fluorescence from the yeasts was insensitive to changes in the extracellular perfusate; no change occurred when the pH of the perfusing BSS was reduced from 7.0 to 6.0. The response of phagosomal pH to weak bases was far less marked than that of the lysosomes. Incorporation of 10 mM ammonium chloride in the BSS perfusing the fluorescence cuvette had little effect upon the reduction of pH of the phagosomes during the first 30 min after uptake of fluorescent yeasts. However, 0.5–1 h after uptake, addition of ammonium chloride or chloroquine reduced the steady fall in phagosomal pH and sometimes caused a slow increase (Fig. 8).

Effect of Polyanions on Phagosomal pH

Macrophages were preincubated with COAM (200 μg/ml, 24–44 h), and after uptake of antibody-coated, live or heat-killed yeasts, P-LF was found to be inhibited (90% inhibition) as previously described (3). There was no obvious impairment of phagocytic activity. In four experiments, the pH of the yeast phagosome was monitored after rapid rewarming of macrophages bearing FITC-labeled yeasts prebound at 4°C (20 min). There was no significant alteration of the phagosomal pH kinetics of Fig. 9, despite the severe deficiency of P-LF demonstrated by parallel fluorescence microscope studies in the same batch of cells. Further, both live and heat-killed yeasts

TABLE I

Fluorescence Polarization of FITC and FITC-Dextran Conjugates at 37°C

		Polarization	
Glycerol	Viscosity	FITC	FITC-Dextran
% *wt/wt*	*cp*	*P*	
100	450	0.4	0.41
92	113	—	0.36
79	27	—	0.12
40	2.4	—	0.11
9	0.9	—	0.08
0	0.62	0.06	0.07

Measurements were made at pH 7.4 in phosphate-buffered saline/glycerol mixtures at high fluorophore dilution (~1 μg/ml).

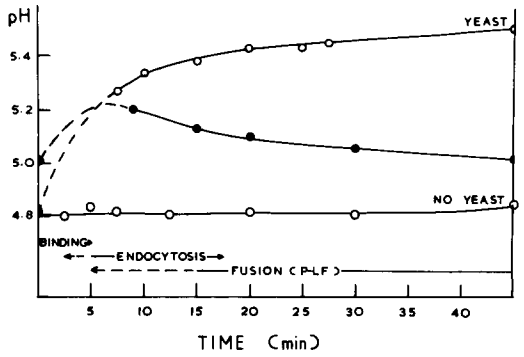

FIGURE 6 The response of lysosomal pH to phagocytosis. Macrophages were exposed to yeast cells for 5 min or to BSS alone. The pH after incubation was followed in the fluorimeter (○). Lysosomes containing COAM in addition to FD40 responded as in (●). The approximate time-scale of observed endocytic processes is also indicated.

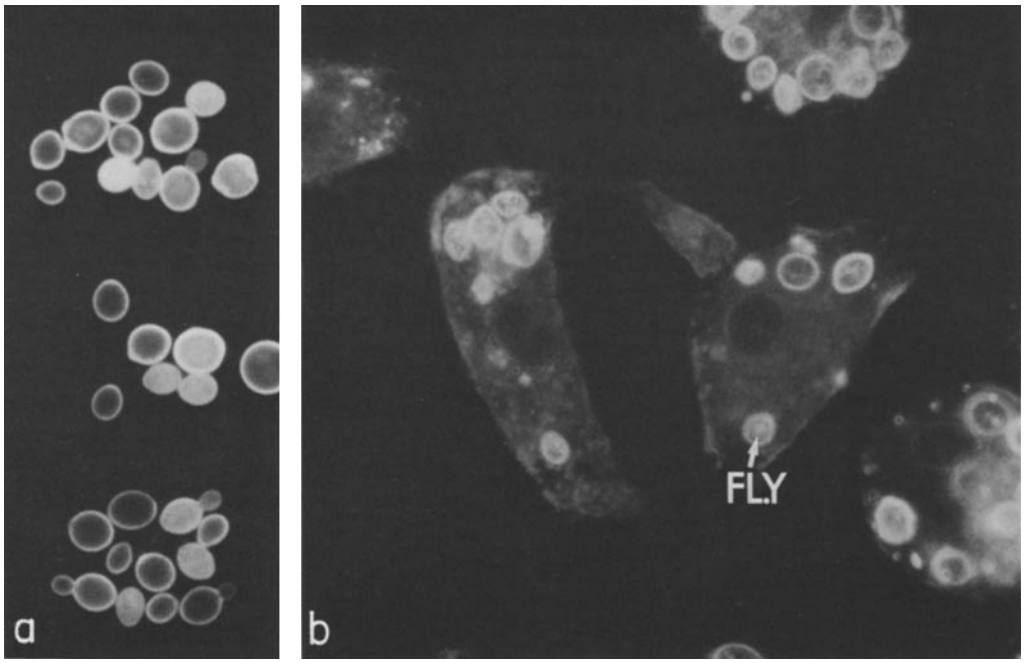

FIGURE 7 (*a*) Surface-labeled fluorescent yeasts produced by carbodiimide coupling of fluorescein amine. × 1,350. (*b*) Macrophages with phagocytosed fluorescent yeasts (*FL.Y*) after incubation as described in the text. × 1,350.

gave rise to the same pattern of pH change within the phagocytic vacuoles.

Phagocytosis of Indicator-stained Target

Earlier investigators (5) employed indicator dyes adsorbed to heat-killed yeasts to estimate phagosome pH. However, they did not commence observation until 10 min after exposure to the leukocytes. Although the effect of factors other than pH (e.g., granule proteins) upon intracellular dyes is not clear, we considered that this technique might provide independent evidence of a transient pH increase in phagosomes. We stained heat-killed yeasts with neutral red (yellow above pH 7.0) and bromothymol blue (green at pH 6.5 and blue at pH 7.5). The extracellular BSS was buffered with 20 mM HEPES at pH 6.5 where neutral red yeasts were red and bromothymol blue yeasts were yellow-green. These yeasts were opsonized with antiyeast IgG and allowed to attach to macrophages for 15 min at 4°C. After warming to room temperature, neutral red yeasts seen entering macrophages turned from red to a pale yellow and returned to a brilliant red within 1 min. Similarly, bromothymol blue yeasts (yellow-green in the BSS) turned blue after entry, then green, and then yellow. These observations of individual vacuoles support the fluorescence observations that the phagosomal pH is first increased from that of the external medium (to at least pH 7.5) and then within 5 min is reduced to a pH <6.5.

DISCUSSION

In this study, pH within the lysosomes and phagosomes of cultured mouse macrophages was continuously measured during P-LF. We found that the average pH of the phagosome was reduced in 15–20 min from that of the external medium to pH 5.4 ± 0.4, while the same average pH (5.4 ± 0.2) was recorded in lysosomes at this time. This result is gratifying to the extent that one might anticipate both spectroscopic probes to monitor the same pH after fusion of all the lysosomes with phagosomes. Complete degranulation of the macrophages, however, did not occur as also noted by others (13); there were always unfused probe-containing lysosomes. In contrast, after 3 h nearly 100 % of phagosomes were seen to contain lysosome-derived fluorescence. During the intial 5-min exposure of the macrophage-carrying cover slip to the fluorescent yeast suspension, the lysosomal pH apparently increased, even when the same lysosomes contained a polyanion that effectively prevented their fusion with phagosomes. We have tentatively attributed this pH change to a metabolic effect (3), because cellular ATP is being drawn upon during the phagocytosis of yeast. After phagocytosis was complete, the lysosomal pH of polyanion-treated cells returned to its original level, whereas the lysosomal pH of untreated cells continued to rise, presumably because of transfer of fluorescent dextran to the higher pH environment of the phagosomes.

We realized that the environment subject to pH determinations was poorly defined during the first 15 min because yeast cells were in three possible states after the initial 5-min incubation: bound to the cell surface, actively entering the cell, and (to a small extent) already within the vacuolar apparatus. For this reason we attempted to synchronize the phagocytosis of yeast by prebinding the fluorescent organisms at 4°C. Rapid rewarming of the monolayer to 37°C then provided a better-defined start for the kinetic observations. When this was done, not only did we measure initially the pH of the medium, but within 1 min of warming, the fluorescence from the bound yeast increased, apparently indicating a pH higher than the external medium (up to pH 7.75). After 5 min, the pH decreased as in the experiments performed entirely at 37°C. This unexpected result was obtained consistently when the endocytosed yeast was labeled either with fluorescent antibody or directly with FITC, or even when FITC-labeled *Staphylococcus aureus* was substituted as a phagocytic target. We do not think that the fluorescence increase was engendered by the warming step. The spread morphology of the cells was not obviously altered by the cooling/warming procedure, and cells alone produced

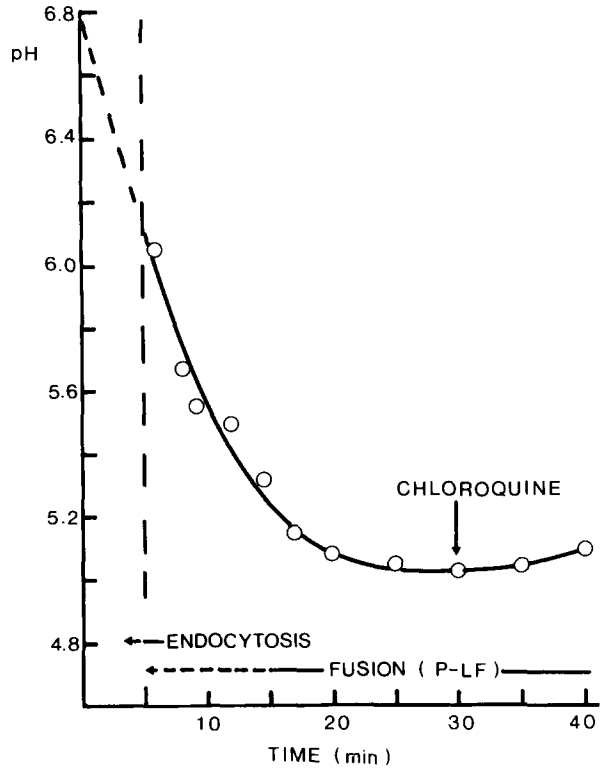

FIGURE 8 The pH of phagocytic vacuoles measured by fluorescein conjugated to *S. cerevisiae*. After a 5-min preincubation of yeasts and monolayer cells (vertical broken line), the monolayer was vigorously washed and transferred to the fluorimeter. The fluorescence cuvette was continuously perfused during the observations. pH was determined at intervals by recording the F_{490}/F_{450} ratio and confirmed at the beginning and the end by scanning the entire excitation spectrum. After 30 min, 100 μM chloroquine was perfused through the cuvette. In controls, (not shown) the pH remained steady or continued to decrease slowly at this time.

no significant background fluorescence changes during rewarming and subsequent incubation. Moreover, performing the same experiment at ambient temperature with a 2-min exposure of the monolayer to yeasts also allowed us to record an apparent pH increase. Whether the experiment was performed at 37°C or 4°C, 5 μM cytochalasin considerably delayed and reduced the magnitude of pH changes recorded by bound, fluorescent yeast although the yeast was ultimately phagocytosed and the pH slowly decreased. Neither the early rise nor the subsequent acidification of the vacuolar pH could have been a result of metabolic activity of the yeast, because the same changes were equally observed with viable and heat-killed organisms.

At present we interpret the increase in fluorescence that occurred within seconds of warming macrophages with surface-bound yeast as a net rise in pH of the vacuoles associated with phagocytosis. However, although we cannot now imagine what other enviromental changes could affect the fluorophore in this manner, we cannot exclude the possibility of fluorescence enhancement by a process other than pH change. It is worth noting that the kinetics of the transient pH rise coincide with those of the respiratory burst of leukocytes, and, in neutrophils, with the degranulation of vesicles containing enzymes with an alkaline pH optimum (2, 14).

It is important to make clear the limitations of the present technique. The pH values recorded represent weighted averages over the few thousand cells exposed within the measurement area of the fluorimeter. If the pH within individual vacuoles is heterogeneous with respect to time, as suggested by the approximate observations of Jacques and Bainton (5), then the fluorescence method will always be biased toward a pH higher than the true average. This arises because of the steepness of the acid quenching of fluorescein emission. During the period of endocytosis of bound yeasts, the acidity of internal vacuoles was certainly masked by the high and constant fluorescence of those still exposed to the external medium. A calculation showed that, supposing the internal pH to be 4.5 (the lowest likely), this value would be significantly altered if >5% of the total fluorescein were external. These observations mean that the observed rate of acidification of the phagocytic vacuoles and the final recorded pH are likely to be underestimates and overestimates of the true average values and make our observation of an overall transient pH rise all the more significant. The progress of pH change within a given vacuole could be quite different from our average.

We have previously directly demonstrated that polyanions inhibit P-LF in cultured macrophages (3). It is therefore interesting that P-LF-suppressing levels of a polyanion (COAM) were without effect upon the pH changes that occur within yeast phagosomes for at least 15–20 min after phagocytosis. Our previous studies indicated that over longer periods there may be some reduction in the capacity of the cell to produce acid phagosomes. It is difficult to observe transfer of FD40 from secondary lysosomes to phagosomes before 15 min, but P-LF becomes obvious in normal cells thereafter. However, the most rapid pH changes occur within 15 min.

It therefore seems likely that phagosomal fusion with a large complement of the macrophage granules (those that become labeled by a variety of exogenous macromolecules or permeant dyes such as acridine orange) is not required for the early changes in phagosomal pH. In this respect it may be significant that we do not observe improvement in intracellular yeast survival with polyanion inhibitors of P-LF. Thus it remains an open question whether the early regulation of phagosome pH is a property intrinsic to the unfused phagosomal membrane or is conferred by primary lysosomes or other granules that are

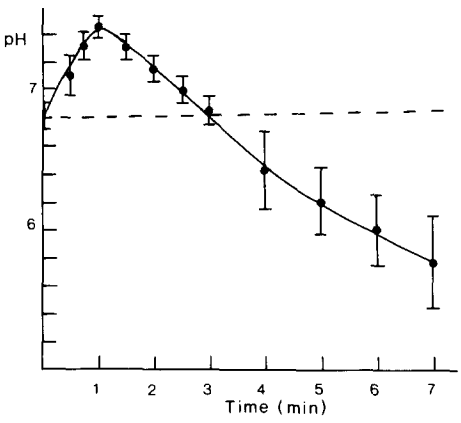

FIGURE 9 The pH of phagocytic vacuoles measured by FITC-anti-yeast antibody bound to boiled *S. cerevisiae*. The IgG-coated yeasts were prebound by exposure to the macrophage monolayer at 4°C for 20 min. Phagocytosis was initiated by rapid transfer to the fluorescence cuvette perfused with BSS at 37°C. The results are the mean of three experiments including both live and boiled organisms. The horizontal broken line represents the pH of the perfusing BSS.

transparent to our present labeling techniques. These observations now raise the interesting question of whether the survival of microorganisms possessing natural, presumably phagosome-directed P-LF inhibition properties (e.g., *Mycobacterium tuberculosis*) results in an ability to efficiently prevent pH changes within the unfused phagosome. This point is currently under investigation.

Using a lysosomal fluorescence probe, we confirmed the pH measurements first made by Ohkuma and Poole (1). In addition we found an identical resting pH in BHK cell lysosomes (pH 4.8). In the electron microscope, macrophage lysosomes are small with a moderately dense matrix and it might be anticipated that the motion of a high molecular weight fluorophore would be restricted within this organelle. We measured the fluorescence polarization of our FD40 probe at high dilution in solutions of known viscosity and found that the conjugate behaved like free FITC, i.e., its molecular motion on a nanosecond time-scale was not significantly altered by conjugation to dextran, which is in line with the random, flexible structure of the polysaccharide. Assuming that the interiorization did not alter the fluorescent lifetime of the probe, motion in the normal intralysosomal environment was relatively restricted by comparison with free solution. Even though lack of lifetime measurements precludes our direct determination of microviscosity, it was notable that polyanions that inhibit P-LF had no effect upon the apparent mobility of the fluorescence probe. By contrast, both weak bases and P-LF itself markedly increased the apparent molecular motion of the probe. The molecular mechanisms by which amines and polyanions perturb P-LF remain unknown, but would appear from these studies to have no common components.

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