

# Nonlysosomal Vesicles (Acidosomes) Are Involved in Phagosome Acidification in *Paramecium*

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**ABSTRACT** Although acidification of phagocytic vacuoles has received a broadened interest with the development of pH-sensitive fluorescent probes to follow the pH changes of vacuoles and acidic vesicles in living cells, the mechanism responsible for the acidification of such vacuoles still remains in doubt. In previous studies of the digestive vacuole system in the ciliate *Paramecium caudatum* we observed and described a unique population of apparently nonlysosomal vesicles that quickly fused with the newly released vacuole before the vacuole became acid and before lysosomes fused with the vacuole. In this paper we report the following: (a) these vesicles, named *acidosomes*, are devoid of acid phosphatase; (b) these vesicles accumulate neutral red as well as acridine orange, two observations that demonstrate their acid content; (c) cytochalasin B given 15 s after exposure of the cells to indicator dye-stained yeast will inhibit the acidification of yeast-containing vacuoles; and that (d) we observed using electron microscopy, that fusion of acidosomes with the vacuole is inhibited by cytochalasin B. We conclude that the mechanism for acidification of phagocytic vacuoles in *Paramecium* resides, at least partially if not entirely, in the acidosomes.

Recent papers that have used pH-sensitive fluorescent probes (1) to follow the time course of the pH changes in phagosomes (2–5) and endosomes (1, 4, 6–8) of various cells suggest that the pH of a vesicle or vacuole that derives from the cell surface often becomes acid before such an organelle fuses with lysosomes. Thus the mechanism for the initial acidification of these organelles would not come from the lysosomes, though some lysosome membranes, such as those of rat liver cells, have now been shown to have a proton pump (9, 10), nor would the acidification in these cases result from the digestive processes that would typically follow lysosome fusion. In *Paramecium* it has long been known that the pH of the digestive vacuole falls quickly following the release of the vacuole from the oral region (11, 12). We recently reported a detailed study of the time-course of the pH changes in the vacuoles of *Paramecium caudatum* grown in axenic medium and showed that the vacuolar pH falls from 7.0 to 3.0 within 5 min (13). By 8 min, the pH begins to rise rapidly to neutrality. The initial acidification parallels the early rapid vacuole condensation (13), while the rise in pH at 8 min corresponds to the approximate time when vacuoles first fuse with lysosomes (14) and acquire acid phosphatase activity (15).

We have also described a population of rather large vesicles (up to 1  $\mu\text{m}$  in diameter), which we termed phagosomal fusion

vesicles (PFVs)<sup>1</sup> (16, 17). These PFVs were observed to bind to the forming digestive vacuole membrane, but they do not fuse with the vacuole until the vacuole has been released from the oral region of the cell and has moved a distance of some 30  $\mu\text{m}$  to the posterior end of the cell. By 15 to 30 s these PFVs have fused with the vacuole. Because these PFVs fuse with digestive vacuoles around the time the vacuolar pH begins to fall, we investigated their potential role in vacuole acidification.

## MATERIALS AND METHODS

For bright-field microscopy, log-phase *P. caudatum* cells growing in an axenic medium at pH 7.0 (18) were concentrated in nylon cloth before incubating for ~8 min in neutral red (0.05 mg/ml axenic medium). Cells were washed several times with fresh axenic medium and allowed to stand for 10 min or longer. Cells were then observed and photographed using a Zeiss microscope after their swimming had been impeded by the pressure of the coverslip. This pressure had no other observable effects and normal cellular activities such as the filling and emptying of the contractile vacuoles and the rapid posterior movement of newly formed digestive vacuoles continued.

For fluorescence microscopy, concentrated cells were incubated in acridine orange (50  $\mu\text{g}/\text{ml}$ ) for 1.5 min. The cells were then washed free of acridine orange with fresh axenic medium and observed using a Zeiss epifluorescence microscope.

<sup>1</sup> *Abbreviations used in this paper:* CB, cytochalasin B; DMSO, dimethylsulfoxide; and PFV, phagosomal fusion vesicles.

To test the effects of cytochalasin B (CB) on vacuole acidification, we first placed a population of cells in medium containing heat-killed, bromocresol green-stained yeast for 15 s before the addition of cytochalasin B (CB) (at 143  $\mu\text{g}/\text{ml}$  in a final concentration of 0.4% dimethylsulfoxide [DMSO], vol/vol). (The typical fluorescence-probe techniques used by others are not useable below pH 4 such as is attained in digestive vacuoles of *Paramecium*.) Half of the cells formed at least one digestive vacuole during this pre-CB pulse. At predetermined intervals aliquots of cells were removed, spread on albuminized slides, and air dried. Air drying required roughly 2 min. The digestive vacuoles containing yeast were then scored for their colors being careful to exclude those vacuoles near to and perhaps still continuous with the oral region. Bromocresol green-stained yeast were blue at pH 7 or above, blue green at pH 6 to 6.5, green at pH 4.5 to 5.5, and yellow at pH 4 or lower (13). Colors were scored within 4 h of drying, since the yeast-containing vacuoles reverted back to blue when allowed to stand overnight. Control cells were placed in axenic medium containing yeast and 0.4% DMSO without CB.

For electron microscopy (EM) cells were transferred to axenic medium which contained both polystyrene latex beads (to label the vacuoles for EM) and CB (only CB was omitted in control cells) for periods ranging from 0.25 to 30 min before fixation. Latex beads were used in place of yeast since *Paramecium* takes up these smaller particles more readily than yeast. Latex beads were suspended in axenic (nutrient) medium to ensure that vacuole processing would be unaffected. Processing for EM was carried out as previously described (19). The Gomori technique (20) was used to show the presence of acid phosphatase.

## RESULTS

### *Phagosome Fusion Vesicles*

PFV's are defined as the large vesicles that (a) specifically associate with the nascent vacuole membrane, (b) have an irregular shape, and (c) contain very little detectable material in electron micrographs (16). These vesicles are illustrated in Fig. 1 surrounding a nascent vacuole. In this experiment we exposed cells for a short pulse in horseradish peroxidase. The electron-opaque horseradish peroxidase-reaction product following incubation in  $\text{H}_2\text{O}_2$  and diaminobenzidine can be seen lining the luminal surface of the vacuole and adsorbed to the latex beads. The absence of horseradish peroxidase-reaction product in these PFVs lying against the vacuole membrane indicates there had been no openings between these PFVs and the vacuole.

### *Accumulation of Weak Bases*

Neutral red was distributed in a punctate pattern throughout the cell and around certain digestive vacuoles. Particularly prominent were the neutral red-containing vesicles close to the forming digestive vacuole (Fig. 2a). These vesicles were observed to remain near the vacuole's surface as the released vacuole moved to the cell's posterior end (Fig. 2a).

Acridine orange also became concentrated in vesicles giving a similar punctate pattern as seen following neutral red exposure. The vesicles around the developing vacuoles (Fig. 2b) and newly released vacuoles (Fig. 2c) were especially bright orange. As a digestive vacuole was forming it acquired a coat of these vesicles which moved posteriorly with the released vacuole (Fig. 2, b and c). Since the PFVs are the only vesicles found to line the nascent vacuoles in electron microscopy (Fig. 1), we conclude that the vesicles containing either neutral red or acridine orange that border nascent vacuoles are the PFVs.

### *CB Effects*

We also investigated the effects of CB on the acidification of digestive vacuoles. Having observed that microfilaments occupy the space between the PFVs and vacuole membranes (16), we reasoned that this microfilament-active drug might

affect the fusion of these vesicles. If the PFVs were indeed responsible for vacuole acidification, blocking their fusion with the newly released vacuoles should prevent acidification; this drug has been shown to reduce pH changes in macrophage (2) and neutrophil (3) phagosomes. In control cells placed in bromocresol green-stained yeast and 0.4% DMSO some 70% of the yeast-containing vacuoles became blue-green (pH 6–6.5), green (pH 4.5–5.5), or yellow (pH  $\leq 4$ ) within 6 min (upper curve, Fig. 3). In cells treated with CB only 10 percent of the yeast-containing vacuoles formed during the 15-s pre-CB pulse became blue-green and none became green or yellow during the same time period (lower curve, Fig. 3). Thin sections of CB-treated cells showed the labeled vacuoles that were separated from the oral region to have PFVs remaining around them (Fig. 4b) long after the PFVs would have fused in control cells (Fig. 4a). Serial sections were studied to determine that the PFVs were unfused. This concentration of CB stopped vacuole release (but not increase in diameter) for several minutes after which the vacuole number per cell increased slowly.

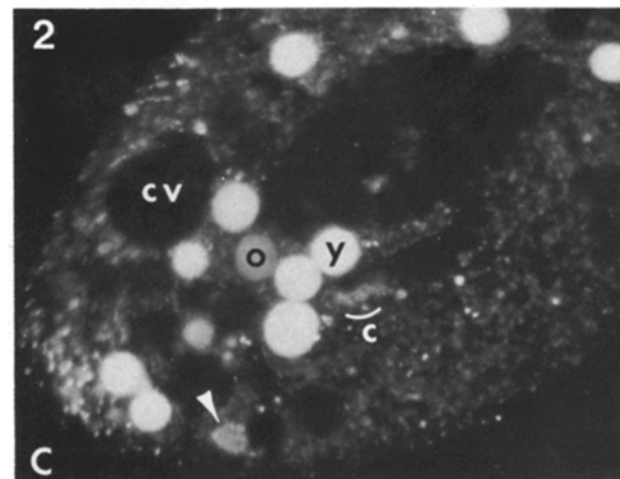
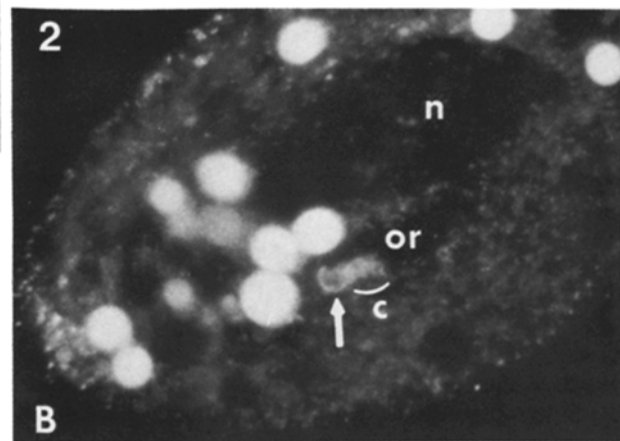
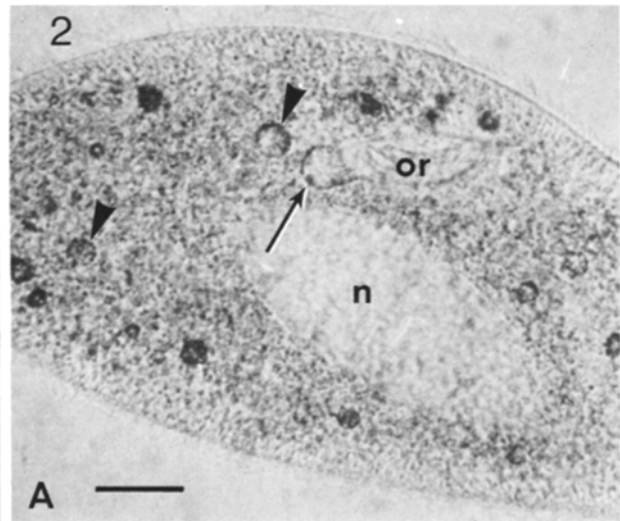
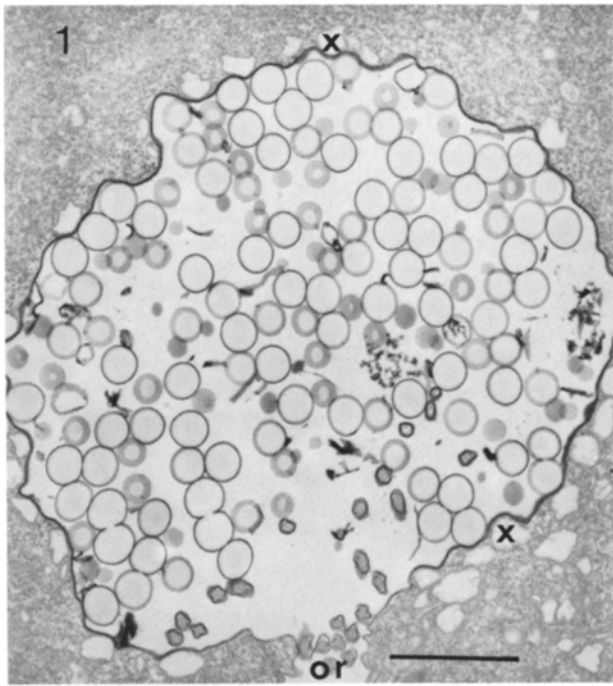
### *Acid Phosphatase Cytochemistry*

Acid phosphatase reaction product was found in some digestive vacuoles, in lysosomes and smaller vesicles in the cytoplasm as previously reported (12, 15, 21). However, no reaction product was found in the PFVs either in the cytopharynx region or around the newly formed digestive vacuole (Fig. 5). Reaction product was never found in any digestive vacuole to which PFVs were associated.

## DISCUSSION

The observations reported here showed that the PFVs found around the developing and newly released vacuoles are themselves acid, for they accumulate both neutral red and acridine orange. Neutral red and acridine orange, which are weak bases, have been shown to accumulate in vesicles such as lysosomes that have an acid pH (22–25). This is presumably due to diminished membrane permeability by the protonated forms of these bases (6). The observation that acridine orange changes in color from green to yellow to red orange when this weak base becomes increasingly concentrated in an acid environment has previously been used to identify acid vesicles in cells (25) as well as to follow phagosome-lysosome fusion in macrophages (26). Our results confirm the observation of Mast (11) that neutral red granules (which seem to us to encompass both lysosomes and PFVs [16]) are located around developing vacuoles and that they move with the vacuole to the cell's posterior pole. Furthermore, our studies have shown that these acidic PFVs fuse with the vacuole before the vacuole becomes acid (13, 16, 17). Thus by fusing with newly released digestive vacuoles these PFVs will contribute their load of protons to the vacuole as well as the mechanism whereby the PFVs can accumulate these ions. This is analogous to the addition of proton pump-containing vesicles to the luminal surface of epithelial cells in turtle bladders (25). We conclude that these PFVs are responsible, at least in part, for bringing about the acidification of digestive vacuoles in *P. caudatum* and we propose that these now be called *acidosomes*.

To our knowledge, this is the first report of nonlysosomal vesicles being involved in acidification of phagocytic vacuoles. That these vesicles are nonlysosomal is deduced from the facts that (a) they fuse with the vacuole several minutes before the



FIGURES 1 and 2 Fig. 1: An electron micrograph of a digestive vacuole still attached to the oral region (*or*) of *Paramecium caudatum*. This cell had been pulsed 15 s with polystyrene latex beads (0.8  $\mu\text{m}$  diam) and horseradish peroxidase whose reaction product following incubation in 3,3'-diaminobenzidine and  $\text{H}_2\text{O}_2$  formed the electron-opaque deposit lining the luminal side of the vacuole membrane and adsorbed to the latex beads. Acidosomes (previously called PFVs) (*x*) are bound to but are not fused with the forming digestive vacuole membrane as indicated by their lack of horseradish peroxidase-reaction product. Bar, 2  $\mu\text{m}$ .  $\times 8,400$ . Fig. 2: Wet mounts of living *P. caudatum* incubated in neutral red (*A*) and acridine orange (*B* and *C*). Both compounds accumulated in a punctate pattern in the cells and were found within granules that rim the forming digestive vacuole (arrows, *A* and *B*) and these granules moved with the released vacuole toward the posterior pole of the cell (arrowheads, *A* and *C*). The dark granules bordering the forming and newly released vacuoles in *A* were red and the rims of similar vacuoles in *B* and *C* fluoresced bright orange demonstrating that both neutral red and acridine orange were concentrated in the acidosomes that line these vacuoles and the cytopharynx (*c*) area. Both *B* and *C* are of the same cell; the developing vacuole in *B* (arrow) is seen in *C* (arrowhead) a few seconds after its release from the oral region (*or*). Other digestive vacuoles fluoresced yellow (*y*) or orange (*o*), depending on their pH and consequent concentration of acridine orange, or not at all (unlabeled circular dark profiles). *n*, macronucleus; *cv*, contractile vacuole. Black and white pictures were reproduced from Ectachrome 400 color film of living cells with original exposure times of 1/60 s (*A*) and 4 s (*B* and *C*). The long exposure time required in *B* and *C* coupled with cyclosis of the cytoplasm resulted in some blurring and in the lack of definition of individual vesicles around developing and newly released vacuoles. The apparent fluorescence within the developing vacuole in *B* is most likely an artifact caused by the bright orange acidosomes surrounding the vacuole but above and below the plane of focus. Bar, 10  $\mu\text{m}$ .  $\times 1,200$ .

vacuole acquires acid phosphatase activity (15), (*b*) cytochemical studies show no acid phosphatase reaction product in these vesicles, and (*c*) morphologically they do not resemble lysosomes. Acidosomes contain neither the prominent glycocalyx on their luminal membrane surface nor the paracrystalline matrix material that is characteristic of lysosomes in *P.*

*caudatum* (18, 27). Furthermore lysosomes concentrate only around the condensed and most acidic vacuoles ( $\sim 5$  min old) and fuse only with the vacuoles that are  $\geq 8$  min old (14). These vacuoles then become acid phosphatase positive (15).

Caution should be exercised in generalizing this finding to other cell types. Whereas the phagosomal membrane in *Par-*

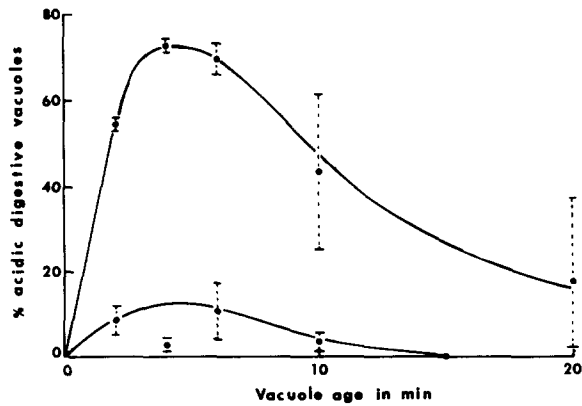
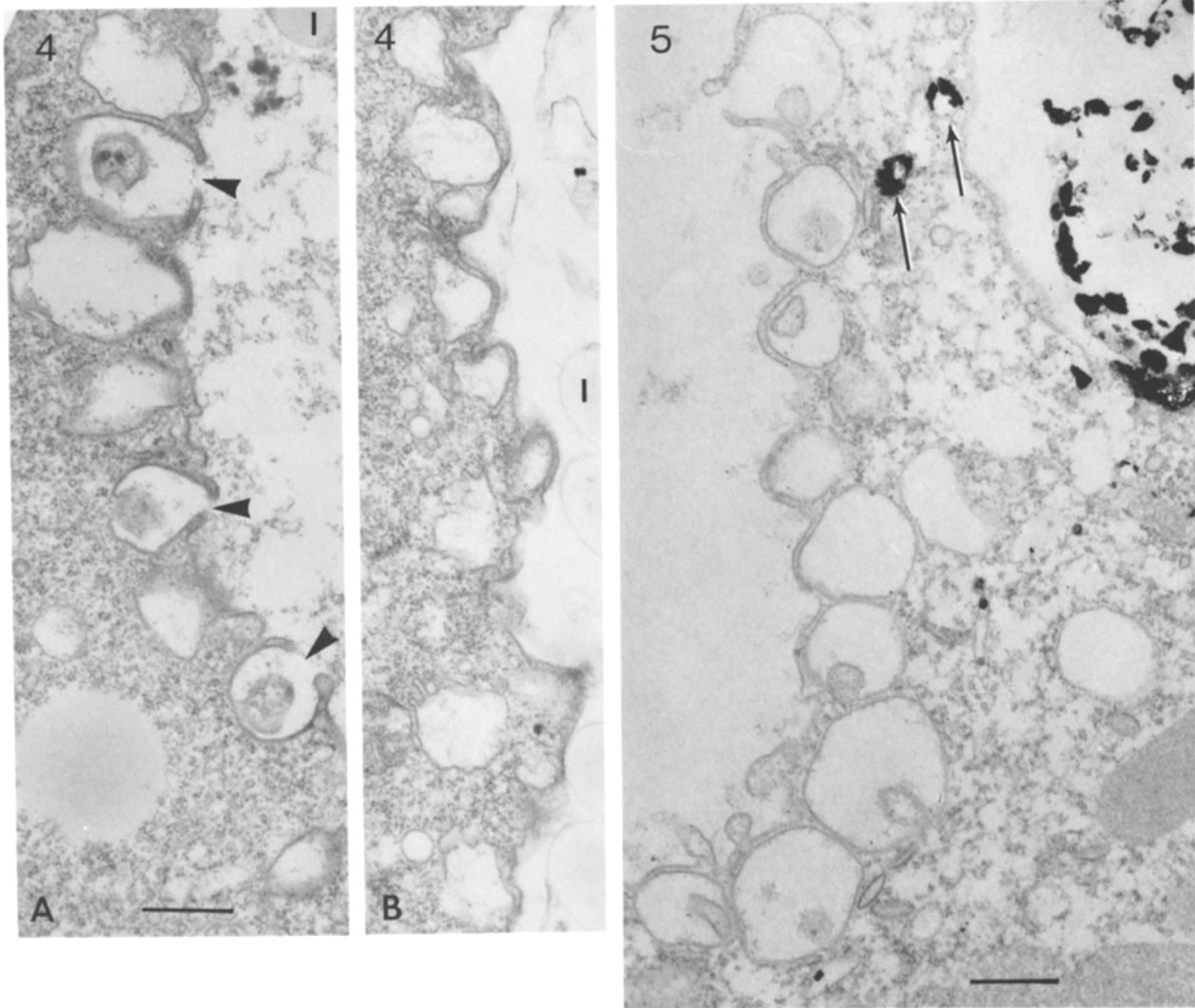


FIGURE 3 Effects of CB on vacuole acidification. Most free digestive vacuoles in control cells (upper curve) incubated in heat-killed, bromocresol green-stained yeast became acid within 3 to 4 min of

*amecium* is derived from a pool of discoidal vesicles (19, 28), the phagosomal membrane in the phagocytic cells of mammals (29) or even in other protozoa such as amebae (30) is derived from the plasma membrane. If the plasma membrane of these other cell types contain proton pumps, their phagosome acidifying mechanisms may be acquired directly from the plasma membrane rather than from a population of vesicles such as the acidosomes. Subsequent investigations are needed to clarify this question.

formation. When treated with CB (lower curve) following a 15 s preexposure to yeast, only 10% of these free vacuoles became acid and then only mildly so (pH ~6.0). During this preexposure some 50% of the cells formed a single vacuole. Results are given as percentage values, mean  $\pm$  SD of two to three experiments and each point represents the average of 50-100 vacuoles. Each time point represents the time (~2 min) an aliquot of cells was spread on a slide and air dried.



FIGURES 4 and 5 Fig. 4: (A) Acidosomes fused (arrowheads) with the membrane of a 30-s-old digestive vacuole containing latex beads (*l*) in a control cell. (B) Acidosomes remained bound to but unfused with a free labeled digestive vacuole that formed while the cell was continuously exposed to CB and latex beads (*l*) for 10 min. The actual time of the release of this vacuole is unknown but only one acidosome was found to be continuous with a vacuole in the five vacuoles studied which had formed during the 10-20-min exposure to 143  $\mu$ g/ml of CB. Sequences of serial sections were followed to establish that the membranes of acidosomes were not continuous with the vacuole membrane. Bar, 0.5  $\mu$ m.  $\times$  25,000. Fig. 5: Portions of two vacuoles are shown, one containing electron-opaque acid phosphatase reaction product and one without. Acidosomes line the margin of the acid phosphatase-negative vacuole and are themselves devoid of acid phosphatase reaction product. The smaller lysosomes (arrows) contain reaction product. Bar, 0.5  $\mu$ m.  $\times$  25,000.

This work was supported by National Science Foundation grants PCM 81 10802 and PCM 82 01700.

Received for publication 8 February 1983, and in revised form 20 April 1983.

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