Prelysosomal Acidic Vacuoles in *Dictyostelium discoideum*

Harish Padh, Malti Lavasa, and Theodore L. Steck

Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois 60637

Abstract. We have examined the ameba *Dictyostelium discoideum* for evidence of a discrete, prelysosomal, acidic receiving compartment in endocytosis. We observed in the cytoplasm abundant round vacuoles with diameters up to 2 μm that concentrated acridine orange by a process inhibited by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). They were therefore taken to be acidic. The vacuoles were observed to fuse nearly quantitatively with primary phagosomes over 30 min and thereby to confer upon them the ability to accumulate acridine orange. The entry into lysosomes of phagocytic cargo occurred later. In the absence of phagocytosis, almost all of the acidic vacuoles rapidly accumulated fluorescent markers that had either been covalently coupled to the cell surface or fed as the soluble dextran conjugate. Therefore, these vacuoles also lie on the pathway of pinocytosis.

A prominent subcellular ATPase activity inhibited by 25 μM NBD-Cl co-distributed on sucrose equilibrium density gradients with vacuoles capable of concentrating acridine orange in vitro. The peak was broad and more buoyant than that bearing lysosomal acid hydrolases, which contained only a minor amount of this ATPase. Also migrating in the buoyant peak were internalized plasma membrane markers; e.g., ^{3}H-galactose had been covalently coupled to the surface of intact cells and allowed to enter pinosomes.

We conclude that in *D. discoideum* an extensive prelysosomal vacuolar compartment provides the proton pumps that acidify both phagosomes and pinosomes.

THAT ingested materials rapidly reach acidic intracellular compartments has been known for a century (for review see Mellman et al., 1986). Classically, these compartments were equated with digestive vacuoles (secondary lysosomes) formed by the fusion of internalized plasma membrane with lysosomes (Steinman et al., 1983). It has become clear in recent years, however, that, in the case of pinocytosis and receptor-mediated endocytosis, incoming vesicles first enter an intervening acidic compartment from which plasma membrane molecules are diverted back to the cell surface while the internalized cargo is delivered to the lysosomes. The acidification is brought about by specific vacuolar proton pumps that are also present in lysosomes (Al-Awqati, 1986; Mellman et al., 1986; Rudnick, 1986).

Although prelysosomal endocytic compartments have been known for many years (de Duve and Wattiaux, 1966), they are not yet well defined. Some have argued that receptor/ligand complexes that have been subjected to endocytosis move directly to the region of the *trans*-Golgi network in receptosomes (Willingham and Pastan, 1984; Pastan and Willingham, 1985). A more prevalent view posits a separate sorting organelle with a distinctive tubulo-vesicular morphology: the endosome or compartment of uncoupling of receptor and ligand (CURL).[1] It is uncertain whether this structure derives its membrane and its powers of acidification from the plasma membrane internalized in the endocytic vesicles themselves (as connoted by the term "endosome") or from a discrete intracellular organelle (as connoted by the term "CURL") (Mellman et al., 1987).

The investigation of prelysosomal processing has not taken full advantage of the third major endocytic pathway which is the phagocytosis of particles. In amebas and macrophages, this process can drive the internalization of three plasma membrane equivalents each hour (Steinman et al., 1983; Thilo, 1985). It has been generally presumed that primary phagosomes fuse directly with lysosomes (Mellman et al., 1986). Indeed, a substantial fraction of the lysosomal membrane in macrophages may be derived from internalized plasma membrane (de Chastellier et al., 1987). Nevertheless, early acidification of phagosomes provides evidence for their prelysosomal processing (Geisow et al., 1981; Allen and Fok, 1983*b*; McNeil et al., 1983). The source of the proton pump in the phagosomes (whether from the plasma membrane, a prelysosomal organelle, or the lysosomes) has not been resolved.

Dictyostelium discoideum is the most primitive eukaryote whose cell biology is known in detail (Loomis, 1982; Spudich, 1987). We now discern abundant prelysosomal vacuoles in this ameba that fuse with and acidify both phagosomes and pinosomes. The data support the hypothesis that the repository of endocytic proton pumps is a discrete, dynamic organelle.

1. *Abbreviations used in this paper*: CURL, compartment of uncoupling of receptor and ligand; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.

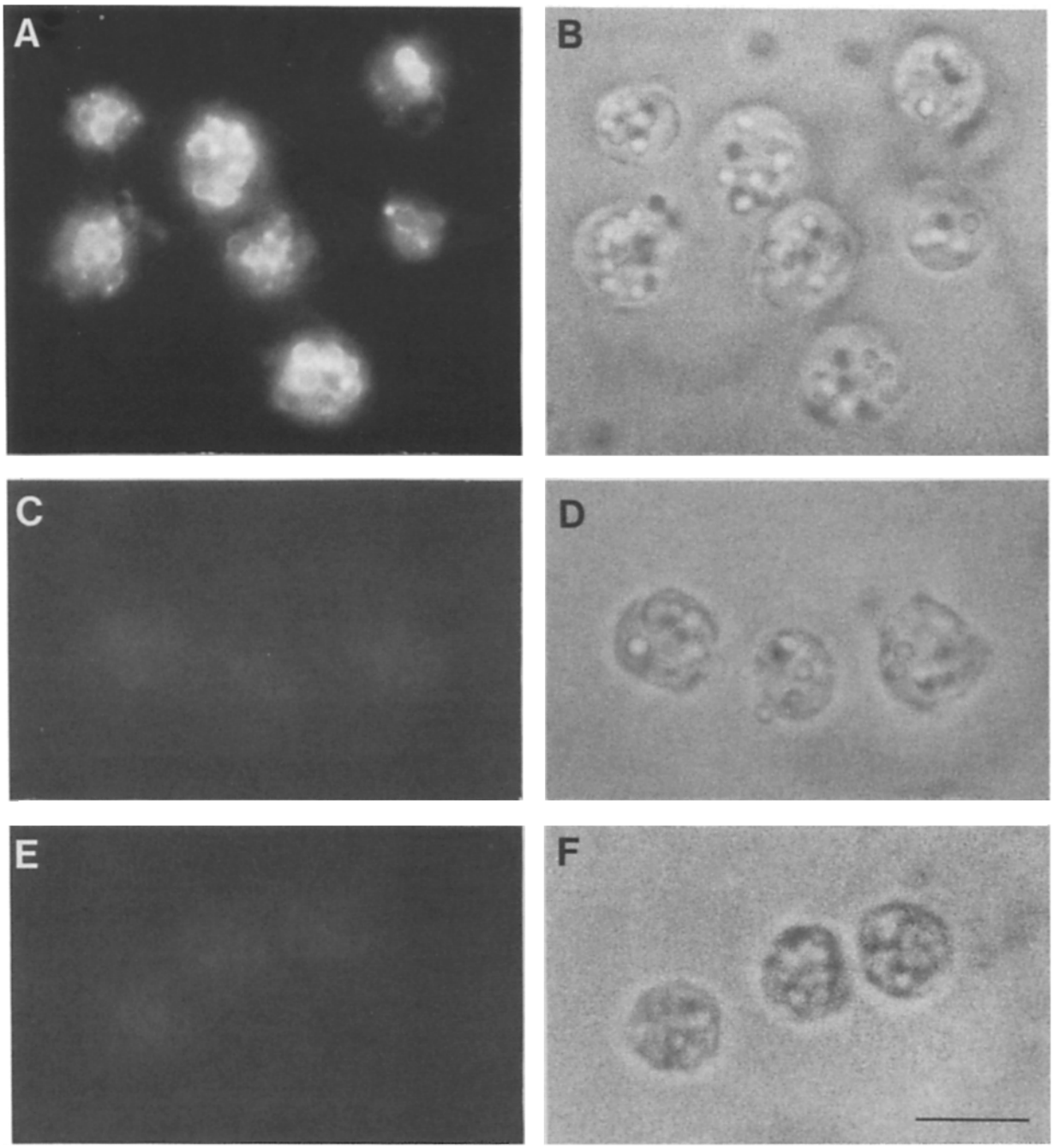

Figure 1. Accumulation of acridine orange in acidic vacuoles in vivo. Cells were washed and resuspended to 5×10^7 ml^{-1} in 150 mM NaCl, 17 mM KP_i (pH 7.5), and 2 mM $MgSO_4$, and gyrated at 200 rpm at 22°C for 15 min. Acridine orange was then added to 3 μM. The cells were photographed 10–25 min later, using a Zeiss fluorescence microscope fitted with a fluorescein filter set and Kodak 2475 recording film (1,000 ASA). (*A* and *B*) Control cells; (*C* and *D*) cells pretreated with 100 μM NBD-Cl for 5 min; (*E* and *F*) cells pretreated with 10 μM nigericin for 5 min. (*A*, *C*, and *E*) Fluorescence micrographs (30-s exposure); (*B*, *D*, and *F*) corresponding phase-contrast micrographs (0.25-s exposure). Bar, 10 μm.

Materials and Methods

Materials

The following biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO): ascorbic acid; 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole(NBD-Cl); FITC-dextran (mol wt 70,000); 2-*N*-morpholinoethane sulfonic acid (titrated to the stated pH with NaOH); acridine orange; and *p*-nitrophenyl-phosphate. Texas red–conjugated dextran (mol wt 70,000) and tetramethyl-rhodamine-5(and-6)isothiocyanate were purchased from Molecular Probes Inc. (Junction City, OR) and [7(*n*)]-^{3}H-cholesterol (10 Ci/mmol) was from Amersham Corp., Arlington Heights, IL). Polycarbonate filters (25-mm

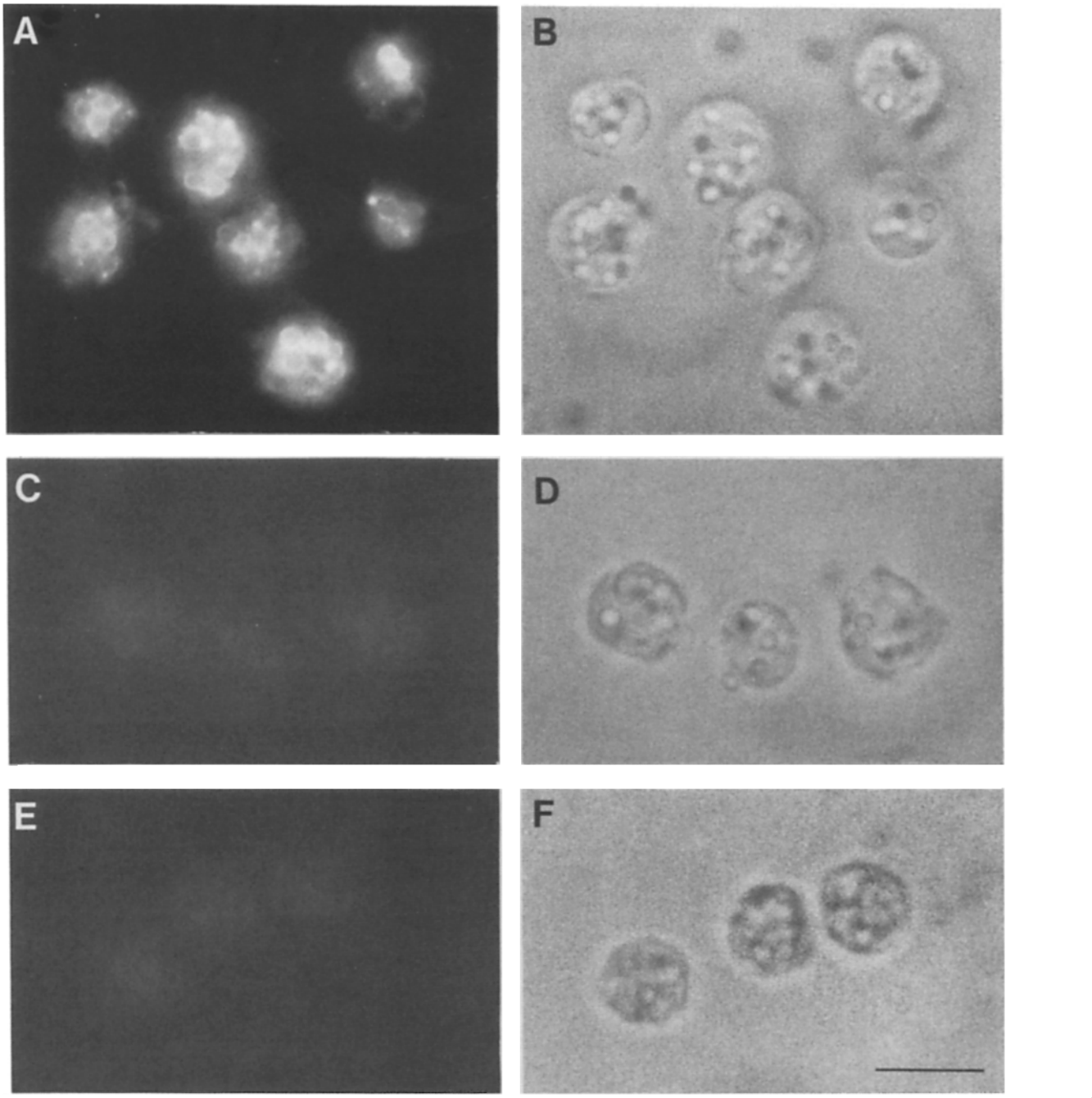

Figure 2. Accumulation of acridine orange in phagosomes. Cells were washed and resuspended to 5×10^7 ml^{-1} in fresh medium. Polystyrene beads of 1.7-μm average diameter were added at 20 beads/cell. After gyration at 22°C for 30 min, the cells were centrifuged to remove free beads and resuspended at 5×10^7 ml^{-1} in 250 mM 2-*N*-morpholino-ethanesulfonic acid-NaOH, pH 7.0. Acridine orange was then added to 3 μM, and the cells were photographed as described in Fig. 1. (*A* and *B*) No treatment; (*C* and *D*) preincubation for 5 min with 100 μM NBD-Cl; (*E* and *F*) preincubation for 5 min with 10 μM nigericin. (*A*, *C*, and *E*) Fluorescence micrographs (30-s exposure); (*B*, *D*, and *F*) corresponding phase-contrast micrographs (0.25-s exposure). Bar, 10 μm.

diameter and 5-μm pores) were obtained from Nuclepore Corp., (Pleasanton, CA). Polystyrene beads with 1.7-μm diameter were procured from Polysciences (Warrington, PA). Other reagents were at least of analytical grade.

Cells

D. discoideum, strain Ax-3 (ATCC 28368), was obtained from the American Type Culture Collection (Rockville, MD) and cultured in HL-5 axenic medium (Sussman, 1987) on a gyrorotary shaker at 200 rpm in a 23°C incubator. Vegetative cells were grown with a doubling time of ~12 h. They were harvested at a density of ~5×10^6 ml^{-1}, pelleted at 400 *g* for 3 min, washed, and resuspended in the indicated buffer. For endocytosis, the cells were swirled with the probes on ice or at room temperature at 200 rpm.

Assays

The P_i generated by ATPase activity was determined as the molybdate complex by optical absorbance measured at 750 nm as described (Serrano, 1978). Unless indicated, the reaction contained (in 350 μl) 50 mM Tris-2-*N*-

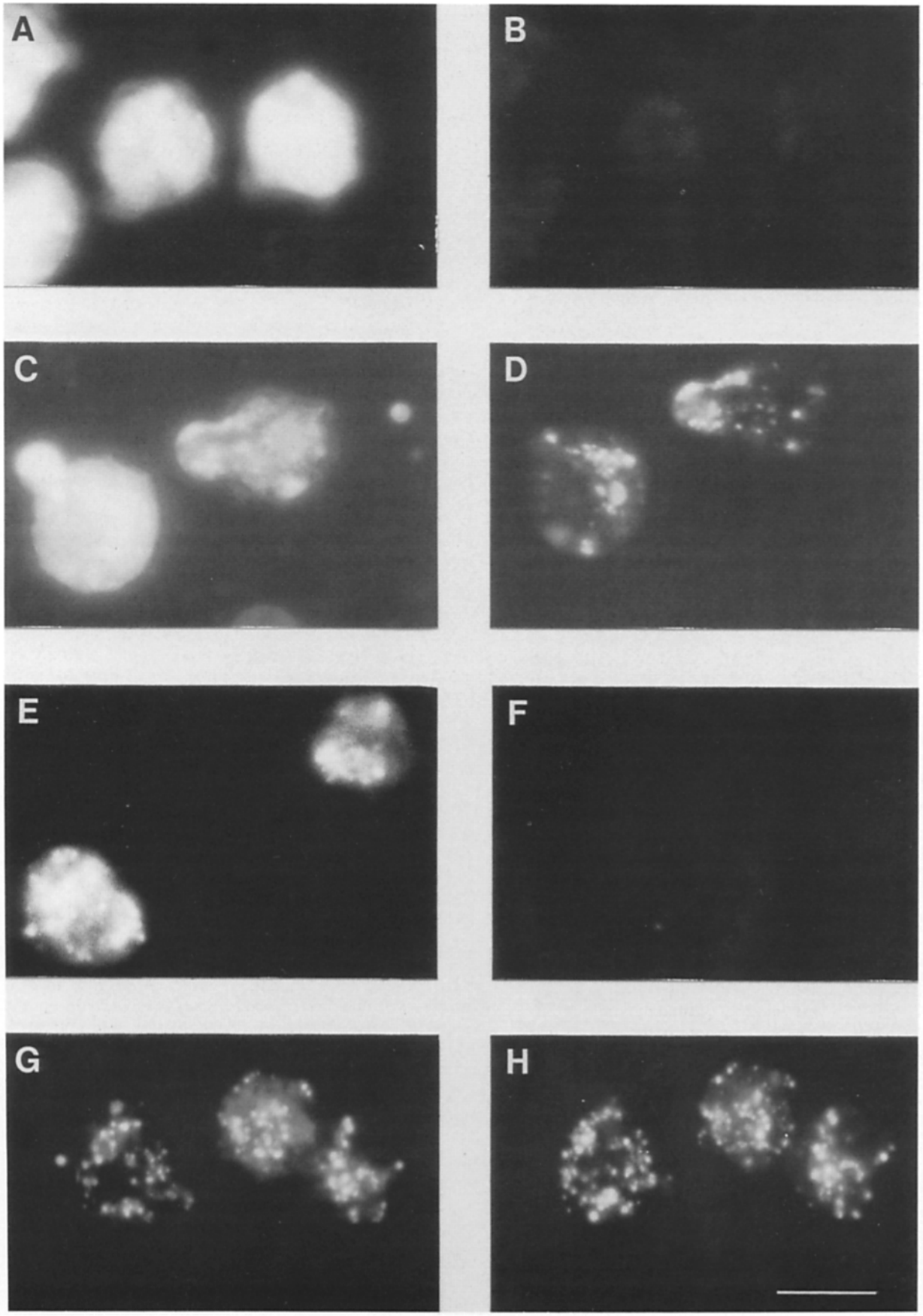

Figure 3. Flux of plasma membrane into acidic vacuoles. Cells were washed and resuspended to 5×10^7 ml^{-1} in 2 ml of ice-cold 17 mM KP_i (pH 6.5) containing 2 mM $MgCl_2$. Tetramethylrhodamine-5(and -6)isothiocyanate was added from DMSO to a final concentration of

morpholino-ethanesulfonic acid (pH 7.0), 5 mM $MgCl_2$, and 10–50 μg membrane protein with or without 25 μM NBD-Cl. Reaction mixtures were preincubated at 30°C for 5 min before the addition of ATP to 2 mM. After 10 min incubation at 30°C, the reaction was terminated by the addition of 0.7 ml of 2% H_2SO_4 containing 0.5% SDS and 0.5% ammonium molybdate, followed by 10 μl of 10% ascorbic acid. Vacuolar H^+-ATPase activity was defined operationally as the difference in the rate of ATP hydrolysis in the presence and absence of 25 μM NBD-Cl (Mellman et al., 1986).

Acid phosphatase activity (Wiener and Ashworth, 1970) and protein (Bradford, 1976) were determined as described.

Results

Visualization of Acidic Vacuoles In Vivo

Acridine orange is a fluorescent tertiary amine that becomes concentrated in the acidic compartments of living cells (Gluck et al., 1982). Intact amebas of Ax-3, an axenic strain of *D. discoideum*, accumulated acridine orange in round vacuoles dispersed in large numbers throughout the cytoplasm (Fig. 1 *A*). The vacuoles ranged in size up to ~2 μm in diameter. They were stained from bright yellow to deep orange, suggesting variation in their pH (Gluck et al., 1982); the cytoplasm was colorless. That these compartments were acidified by a vacuolar ATPase was suggested in Fig. 1, *C* and *E*, which shows the disappearance of their fluorescence upon treatment of the cells with 100 μM NBD-Cl and 10 μM nigericin (Al-Awqati, 1986; Rudnick, 1986). In the presence of NBD-Cl and nigericin, the cytoplasm was stained yellow green by acridine orange, which is consistent with a slightly acidic pH (Fechheimer et al., 1986).

Phagosome Acidification by Vacuoles

As previously reported (Githens and Karnovsky, 1973), vegetative amebas ingested polystyrene beads with a fairly linear time course over a period of 30 min. Treating such cells with acridine orange created a bright rim of fluorescence around the internalized beads which presumably reflected the acidification of the narrow aqueous space under the phagosome membrane (Fig. 2 *A*). The accumulation of stain in the phagosomes was inhibited by NBD-Cl (Fig. 2 *C*) and by nigericin (Fig. 2 *E*).

At the earliest times (~5 min of ingestion), the phagosomes did not take up stain, whereas the aforementioned vacuoles did so. As the rim of phagosomal fluorescence developed over a 30-min time course, the acidic vacuoles became progressively associated with the phagosomes (Fig. 2 *A*), and their numbers dwindled. Ultimately, most of them disappeared, apparently by fusion with the phagosomes. This suggests that a principal function of these vacuoles is to acidify the endocytic/digestive circuit.

These results are consistent with preliminary subcellular fractionation studies. We fed polystyrene beads to *D. discoideum*, homogenized aliquots of cells at intervals, and isolated the buoyant phagosome fraction isopyknically on sucrose barriers of 1.05 g/cc. Initially, the bead-containing fraction had little NBD-Cl–sensitive ATPase, but, during an hour, its enzyme activity increased severalfold (Swaisgood, M. H., H. Padh, and T. L. Steck, unpublished observations).

Flux of Plasma Membranes into Acidic Vacuoles

The plasma membranes of intact amebas were prelabeled covalently with tetramethylrhodamine. Initially, these cells bore intense fluorescence exclusively on their surfaces (Fig. 3 *A*). However, it rapidly entered the cytoplasm in a patchy distribution (Fig. 3 *C*, *right cell*). After 30 min of further incubation, most of the label had moved from the cell surface to a profusion of vacuoles distributed throughout the cytoplasm (Fig. 3, *E* and *G*). That the internalized label had entered almost all of the acidic vacuoles in the cytoplasm was demonstrated by its co-distribution with acridine orange (compare Fig. 3, *G* and *H*). (Fig. 3, *B* and *F*, shows that the fluorescence of the rhodamine did not penetrate the fluorescein filters; the converse was also demonstrated but is not shown.)

It seems that the acidic vacuoles received plasma membranes in the absence of particle ingestion presumably by fusion with pinosomes.

Flux of Ingested Dextran through Acidic Vacuoles

That the bulk of the acidic vacuoles in the cytoplasm were in rapid communication with the pinocytic space is demonstrated in Fig. 4. Amebas that had been allowed to briefly ingest Texas red–labeled dextran showed numerous fluorescent cytoplasmic vacuoles (Fig. 4, *A* and *C*); their pattern generally coincided with the vacuoles stained by acridine orange (Fig. 4, *C* and *D*). As the cells containing the labeled dextran were incubated further, the ingested Texas red (perhaps by virtue of its indigestibility) accumulated at a high concentration in a smaller number of larger and intensely stained bodies (Fig. 4, *E* and *G*). These did not generally coincide with those stained by acridine orange (compare Fig. 4, *G* and *H*). That is, most of the vacuoles were stained only by one of the two fluorophores. It seems likely from these data that the undigested dextran conjugate passed from pinosomes through acidic compartments into lysosomes and/or residual bodies.

That nearly all of the acidic vacuoles are engaged in both phagocytosis (Fig. 2) and pinocytosis (Figs. 3 and 4) implies that a single class of organelles constitutes the receiving compartment for both these modes of endocytosis. (Whether or not the endocytosis of folate receptors in *D. discoideum* [see McRobbie, 1986] uses the same pathway is under study.)

Isopyknic Resolution of Acidic Vacuoles from Lysosomes

Homogenates of *D. discoideum* contain abundant vacuoles acidified by a proton pump, as reflected in (*a*) their ability

375 μg/ml (1.5% in DMSO), and the suspension swirled in an ice bath for 1 h. The mixture was then diluted in ice-cold buffer and washed three times. The cells were resuspended in 2 ml of growth medium and rocked for 0 (*A–D*) or 30 min (*E–H*) at room temperature. The cells were then spun and resuspended in the same buffer lacking dye (*A*, *B*, *E*, and *F*) or containing 3 μM acridine orange (*C*, *D*, *G*, and *H*). The cells were photographed using filter sets for Texas red (*A*, *C*, *E*, and *G*) or fluorescein (*B*, *D*, *F*, and *H*) as described in Fig. 1. Bar, 10 μm.

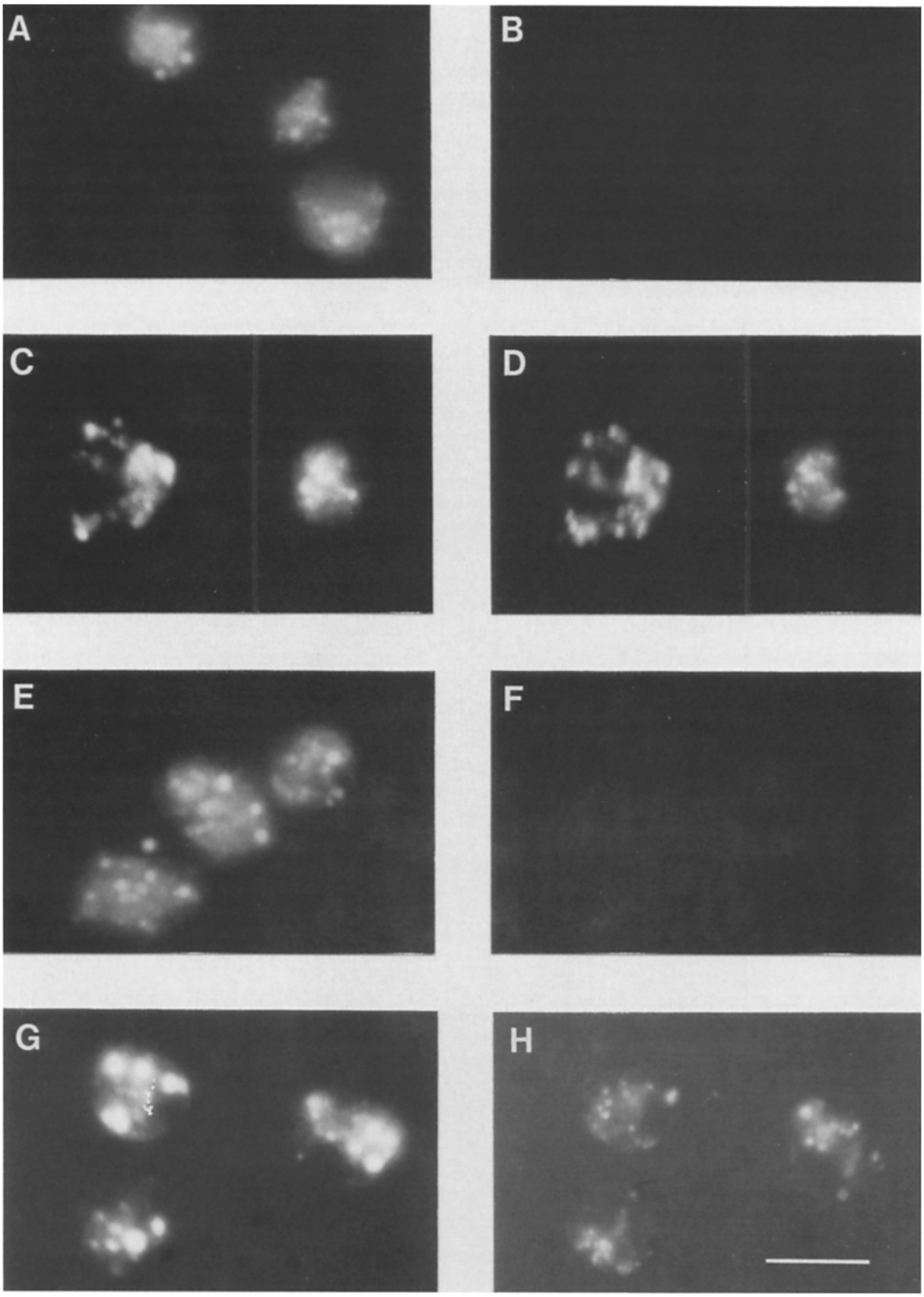

Figure 4. Flux of ingested dextran through acidic vacuoles. Cells were washed and resuspended in fresh growth medium to 5×10^7 ml^{-1}. The suspension was swirled for 30 min at room temperature and dextran conjugated with Texas red added to 2 mg/ml. After 15 min of additional swirling, one aliquot was taken, washed free of external probe, and resuspended in 150 mM NaCl/17 mM KPO_4/2 mM $MgCl_2$ lacking (*A* and *B*) or containing 3 μM acridine orange (*C* and *D*). Meanwhile, a second aliquot was taken from the incubation mixture

to concentrate acridine orange in the presence of MgATP in an NBD-Cl-sensitive fashion; and (*b*) the presence of an NBD-Cl-sensitive, electrogenic membrane ATPase activity (Padh, H., M. Lavasa, and T. L. Steck, manuscript submitted for publication). Upon centrifugation of homogenates to equilibrium on sucrose density gradients, the NBD-Cl-sensitive ATPase was recovered in a broad and complex density profile: there was a major peak at 1.16 g/cc and minor peak at higher density (Fig. 5). In contrast, most of the acid phosphatase activity was at 1.21 g/cc with a minor portion extending into the more buoyant density region and a small soluble fraction at the top of the gradient. Similar patterns were observed with other acid hydrolase markers: *N*-acetylglucosaminidase, β-galactosidase and β-naphthylamidase (not shown). These data demonstrate that most of the H^+-ATPase activity in *D. discoideum* resides in buoyant membranes that are not lysosomes.

Flux of Plasma Membranes into the Acidic Vacuole Fraction

We sought to confirm the implication of the data in Fig. 3 that primary pinosomes merged with the acidic vacuoles. Intact amebas were therefore labeled with ^{3}H-galactose and the cells homogenized either immediately or after a delay of 2 h to permit internalization of the surface label. The isopyknic distribution of the marker on sucrose gradients was then ascertained.

As shown in Fig. 6, the label in the zero-time control sample was mostly in dense membranes that other experiments showed to contain cell surface markers such as exogenous ^{3}H-cholesterol (Padh, H., M. Lavasa, and T. L. Steck, manuscript submitted for publication). A chase for 2 h after labeling caused an increasing fraction of the label to shift to the buoyant region of the gradient, where the NBD-Cl-sensitive ATPase was routinely found. While we cannot rule out the possibility that primary pinosomes are themselves buoyant in spite of the density of their plasma membrane envelope, it seems more plausible that the internalized plasma membrane fused with buoyant acidic vacuoles.

Time Course of Merger of Endocytic and Acid Hydrolase Compartments

Acid phosphatase activity was demonstrated histochemically in the form of numerous moderately sized vacuoles throughout the cytoplasm (not shown, but see Ryter and de Chastellier, 1977; de Chastellier et al., 1983). To determine the kinetics of merger of this compartment with material newly taken up by endocytosis, we prepared red blood cell ghosts into which FITC-dextran, as well as reactants for acid phosphatase histochemistry, had been sealed (Fig. 7, *A*). The ghosts were ingested intact by *D. discoideum* (*B*) but within 30 min, the dye became redistributed into numerous small round bodies resembling the acidic vacuoles described earlier and those in Fig. 7, *C* and *D*. Since the ghosts were apparently broken down in the vacuoles, we infer that the β-glycerophosphate they contained would not be shielded from acid hydrolases. However, only a few of the vacuoles exhibited acid phosphatase activity at this time. During the next hour, a positive reaction for acid phosphatase became detectable in an increasing number of the vacuoles in the form of single small precipitates that excluded the fluorescent stain (Fig. 7, *C* and *D*). The acid phosphatase reaction products were visualized more clearly as phase-dense granules in the phase-contrast microscope, but they were not well photographed and are not shown. The kinetics of their accumulation, however, is plotted in Fig. 8.

We infer from these data that material taken up by phagocytosis reaches the digestive compartment only after acidification. A lag in the confluence of ingested particles and acid phosphatase activity similar to ours was reported for *D. discoideum* by Ryter and de Chastellier (1977). In other studies, acid phosphatase was detected histochemically in vacuoles containing yeast as early as 10–15 min after ingestion (Favard-Sereno et al., 1981; de Chastellier et al., 1983); however, neither a quantitative time course nor the relationship to acidification was reported.

Discussion

How phagosomes become acidified has not been resolved, but three plausible sources of their proton pumps are (*a*) the incoming plasma membranes themselves, (*b*) the recipient lysosomes, and (*c*) intervening acidic vacuoles (Steinman et al., 1983; Mellman et al., 1986).

Our study appears to rule out plasma membranes as likely candidates in *D. discoideum* because the nascent phagosomes are not initially acidic and only become so as they merge with the acidified cytoplasmic vacuoles. This process can be readily visualized in phagosomes (Fig. 2) but not in pinosomes. It may also be that in the case of receptor-mediated endocytosis, the CURL, and not clathrin-coated vesicles derived from plasma membranes, provides the endosomal H^+-ATPase (Anderson et al., 1984; Schmid et al., 1988).

That phagosomes require merger with lysosomes for their acidification also appears unlikely since they do not manifest acid hydrolase activity at an early time (Figs. 7 and 8). Similarly, internalized ligand/receptor complexes and soluble macromolecules in other systems reach acidified compartments before meeting acid hydrolases (Tycko and Maxfield, 1982; Mellman et al., 1986).

The third possibility, fusion of phagosomes with a prelysosomal acidic compartment, is favored by our results and by the current literature. This organelle appears to have the following attributes: (*a*) a membrane/vacuole structure (Figs. 1–3); (*b*) an acidic interior (Figs. 1–4); (*c*) a membrane proton pump of the vacuolar type (Padh, H., M. Lavasa, and T. L. Steck, manuscript submitted for publication); (*d*) rapid fusion with primary phagosomes and pinosomes with the

after 30 min, washed, resuspended in fresh growth medium, and swirled for an additional 3 h. These cells were then washed and exposed to buffer lacking (*E* and *F*) or containing 3 μM acridine orange as above (*G* and *H*). The cells were then photographed (30-s exposure) with a Texas red filter set (*A*, *C*, *E*, and *G*) or a fluorescein filter set (*B*, *D*, *F*, and *H*) as described in Fig. 1. Bar, 10 μm.

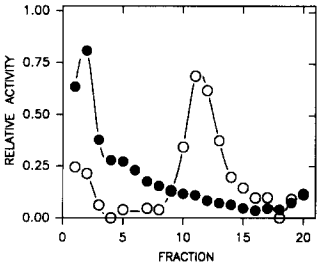

Figure 5. Sucrose density gradient equilibrium distribution of H^+-ATPase and acid phosphatase activities. Cells were washed and resuspended at 2×10^8 ml^{-1} in 5 mM Na glycinate (pH 8.5) containing 100 mM sucrose. Cells were homogenized by a single passage through a pair of 5-μm Nuclepore polycarbonate filters. 1.5 ml of the homogenate was layered on 16 ml of a 25–45% linear sucrose gradient on a cushion of 1 ml 55% sucrose (all sucrose solutions are wt/wt in 5 mM Na glycinate buffer, pH 8.5). After centrifugation in a rotor (model SW27; Beckman Instruments, Inc., Palo Alto, CA) at 25,000 rpm for 3 h at 1°C, 1-ml fractions were collected from the bottom and assayed for acid phosphatase (●) and NBD-Cl sensitive ATPase (○).

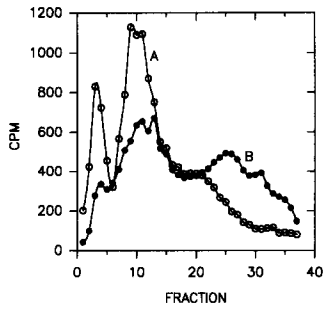

Figure 6. Flux of plasma membranes into the acidic vacuole fraction. Cells were washed and resuspended to 2×10^8 ml^{-1} in 4 ml of 20 mM MOPS-NaOH (pH 6.5) containing 10 mM $MnCl_2$. UDP-^{3}H-galactose (2.2 μM containing 10 μCi in 10 μl) and galactosyl transferase (0.5 U in 10 μl) were added and the suspension swirled in an ice bath for 20 min. 10 ml of ice-cold 20 mM MOPS-NaOH (pH 6.5) was then added, and the cells were washed three times with this buffer. The cells were resuspended in 10 ml of ice-cold growth medium and divided into two portions. One (*A*) was kept on ice while the other (*B*) was first swirled at room temperature for 2 h to allow plasma membrane internalization, and then transferred to ice for another 2 h. The cells were then pelleted and homogenized in 3 ml of 5 mM glycine-NaOH (pH 8.5). Half of each homogenate was layered on a 32-ml linear gradient of 25–45% sucrose (wt/wt) on a 2.5-ml cushion of 55% sucrose in the same buffer. The gradients were spun and fractionated as described in Fig. 5 and the distribution of ^{3}H determined. Note that the buoyant peak of ^{3}H coincided with the H^+-ATPase activity determined in numerous comparable experiments including that shown in Fig. 5.

transient incorporation into the vacuoles of both ingested cargo (Figs. 2 and 4) and plasma membrane markers (Figs. 3 and 6); (*e*) a paucity of acid hydrolases (Fig. 5) but communication with lysosomes (Figs. 7 and 8); and (*f*) a density more buoyant than that of lysosomes and plasma membranes (Figs. 5 and 6).

The cytoplasmic vacuoles described here were thoroughly characterized in previous studies of endocytosis in *D. discoideum*, but their acidification was not examined (Ryter and de Chastellier, 1977; de Chastellier et al., 1983; Schwarz and Thilo, 1983; Thilo, 1985). Their aggregate area was determined to be at least as great as that of the cell surface. When the plasma membrane was externally conjugated with ^{3}H-galactose and allowed to become internalized by either phagocytosis or pinocytosis, the label mixed to equilibrium with the entire population of vacuoles within an hour. Furthermore, the membranes of phagosomes and pinosomes were in rapid equilibration with one another and with the cell surface (de Chastellier et al., 1983). At steady state, more than half of the surface label was internal, and its concentration in the membrane was the same in the vacuoles as in the plasma membrane. Only a small fraction of phagosomes showed histochemical acid hydrolase activity after 30 min (Ryter and de Chastellier, 1977). Acanthameba exhibits similar behavior (for review see Thilo, 1985).

The demonstration that phagocytosis and pinocytosis are coupled through the use of a common pool of internal vacuoles in amebas (Thilo, 1985) agrees with our findings that the bulk of the acridine orange–stained vacuole population in *D. discoideum* can be taken up either by exuberant phagocytosis (Fig. 2) or by pinocytosis (Fig. 3). A shared acidic pathway is strongly indicated.

The equal prevalence of the ^{3}H-galactose label in the surface and internal membranes (de Chastellier et al., 1983) suggests that plasma membrane molecules might make a major contribution to the vacuole membrane. On the other hand, the intramembrane particles in freeze-fractured phagosomes become more abundant and larger than those of the plasma membrane within 15 min of internalization (Favard-Sereno et al., 1981). This suggests that the vacuole membrane has a distinctive composition. The abundant intramembrane particles in these vacuoles might in fact represent the H^+-ATPase identified herein and visualized recently in another system (Brown et al., 1987).

We anticipated finding a large population of vacuoles rich in both H^+-ATPase and acid hydrolases. However, judging from Fig. 5, only a small fraction of the active proton pumps reside in lysosomes in these cells. If the vacuolar H^+-ATPase enters the lysosomes during the transfer of ingested material, its steady-state distribution may reflect an active cycling process favoring its retrieval (Lippincott-Schwartz and Fambrough, 1986). The observed steady-state distribution of the H^+-ATPase may reflect the fact that the amebas were grown on an axenic medium: broth so digestible that it might not demand as many secondary lysosomes as does a natural diet. In that case, the merger of acidic vacuoles with lysosomes could be an induced and regulated rather than a constitutive process. The diffuse buoyant limb associated with the dense peak of acid hydrolases in Fig. 5 could represent such hybrid digestive vacuoles; however, they too have a low complement of H^+-ATPase activity.

A precedent for our findings in *Dictyostelium* comes from *Paramecium*. Prelysosomal acidic vacuoles, called phagosome fusion vesicles (Allen and Fok, 1983*a*) or acidosomes (Allen and Fok, 1983*b*), were observed to fuse with and acidify emergent phagosomes within 3–4 min of their formation at a well-formed gullet, the cytopharynx. The fusion of these acidified vacuoles with lysosomes occurred a few minutes later (Fok et al., 1984). Eventually, the resulting digestive vacuoles underwent transformations suggesting that acid hydrolases and acidosomal and phagosomal membranes were being sorted and recycled (Allen and Fok, 1984; Fok et al., 1984). The latter process could underlie the marked steady-state segregation of the H^+-ATPase from the acid hydrolase compartment in *D. discoideum* (Fig. 5).

While the phagosome-directed acidosomes in *Paramecium* have been considered to be specialized (Mellman et al., 1986), they appear to have counterparts generally. McNeil et al. (1983) observed that the phagosomes of *Ameba proteus* became acidified shortly before their fusion with lysosomes.

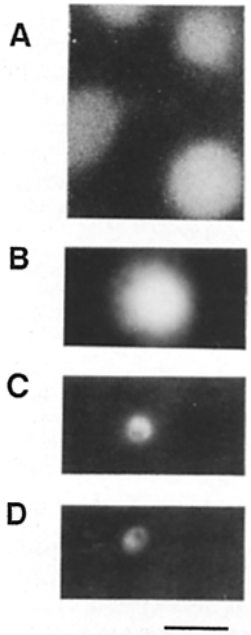

Figure 7. Visualization of the merger of endocytic and acid hydrolase compartments. Unsealed ghosts were prepared from washed red blood cells in 5 mM NaP_i (pH 8) and incubated with the following reactants (final concentrations) for 15 min on ice to allow their complete permeation: 5 mM 2-*N*-morpholino-ethanesulfonic acid-NaOH (pH 6.5), 5 mM $MgSO_4$, 4 mg/ml FITC-dextran, 5 mg/ml β-glycerophosphate, and 2 mg/ml $Pb(NO_3)_2$. The ghosts were then sealed by warming at 37°C for 30 min (see Lieber and Steck, 1982), washed thrice with 10 mM 2-*N*-morpholino-ethanesulfonic acid-NaOH (pH 6.5) plus 5 mM $MgSO_4$, and resuspended in the same buffer. *D. discoideum* were washed and resuspended to 5×10^6 ml^{-1} in growth medium, swirled for 30 min at room temperature, mixed with an equal volume of the loaded ghosts, and swirled. At intervals, aliquots were fixed with 1.25% glutaraldehyde in the cold, and the cells were washed with 100 mM sucrose in 25 mM Na cacodylate buffer (pH 6.8) and resuspended in 10 mg/ml $(NH_4)_2S$ in water for 5 min at room temperature. The cells were washed, resuspended in water, and photographed in the fluorescence microscope as in Fig. 1. (*A*) Input fluorescent ghosts. (*B*) Ghost in a *D. discoideum* phagosome after 10 min of ingestion. (*C* and *D*) Fluorescent vacuoles 90 min after ghost ingestion. Note the single dark dots within the bright circles corresponding to the precipitated acid phosphatase reaction product (see Fig. 8). Bar, 5 μm.

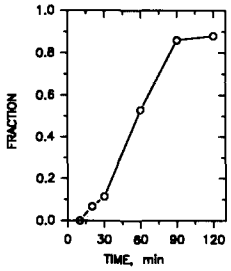

Figure 8. Kinetics of merger of endocytic and acid hydrolase compartments. In the experiment described in Fig. 7, cells were fixed after various times of incubation and the presence of insoluble acid phosphatase reaction product in their fluorescent vacuoles scored using concurrent fluorescent and phase-contrast microscopy. At least 30 fluorescent vacuoles were analyzed for each time-point. The ordinate expresses the fraction of fluorescent vacuoles which contained phase-dense precipitates.

These authors suggested that the plasma membrane was the source of the acidification. In macrophages, newly formed phagosomes were also shown to become acidic before their merger with lysosomes; fusion with unidentified granules was suggested as a mechanism (Geisow et al., 1981). Given all of the data, it seems reasonable to suppose that nascent phagosomes are generally acidified by fusion with specific organelles, the prelysosomal vacuoles, while the CURL is the corresponding organelle specific for receptor-laden endocytic vesicles (Mellman et al., 1986; Mellman et al., 1987).

The prelysosomal acidic compartment in *Dictyostelium* could be regarded as archetypal, in that this protist diverged early in eukaryotic evolution, long before yeasts, ciliates, and ancanthamebas (Sogin et al., 1986). Prelysosomal vacuoles similar to those observed here have been widely observed in mammals as well as in lower eukaryotes (de Duve and Wattiaux, 1966), but their powers of acidification were not investigated until recently. For example, the apical epithelium of the proximal tubules of the kidney takes up proteins by nonspecific adsorptive pinocytosis. The internalized proteins initially enter small clathrin-coated vesicles (Rodman et al., 1984). The proteins soon pass from there into large, round (rather than tubulo-vesicular) vacuoles. These vacuoles slowly deliver the proteins to lysosomes (Straus, 1964; Maunsbach, 1966). As in *Paramecium*, the process is ordered with respect to both timing and intracellular position. The small (primary) vesicles are not conspicuously acidic, whereas the large (secondary) vacuoles are strongly so (Larsson et al., 1987).

Morphometric analysis has demonstrated that the prelysosomal compartment in *D. discoideum* is very extensive (Thilo, 1985), and the present results show that it bears most of the vacuolar H^+-ATPase activity as well (Fig. 5, and Padh, H., M. Lavasa, and T. L. Steck, manuscript submitted for publication). This may also be the case for prelysosomal compartments in at least some mammalian cells. Cultured rat hepatocytes, for example, contain abundant acidic vacuoles that are not lysosomes (Lake et al., 1987). These vacuoles may contribute to the large fraction of hepatocyte membrane ATPase activity, which is not ascribable to lysosomes or mitochondria (Schneider, 1977). Similarly, most of the ATP-dependent, acridine orange quenching activity in Chinese hamster ovary cells is in buoyant vacuoles distinct from lysosomes (Timchak et al., 1986). In cultured fibroblasts, a profusion of small, spherical, acidic vesicles has been distinguished from coated vesicles, lysosomes, and endosomes; the function of these vesicles is unknown (Anderson et al., 1984). Finally, only a small fraction of the membranous H^+-ATPase in human neutrophilic leukocytes is associated with acid hydrolase-rich (azurophilic) granules (Mollinedo et al., 1986). Since some authors have assumed that vacuoles which take up both markers for pinocytosis and acridine orange are secondary lysosomes without testing for their acid hydrolase content, the digestive compartment may in some cases have been overestimated at the expense of the prelysosomal. Valuable in this regard would be quantitative data on the distribution of the NBD-Cl-sensitive H^+-ATPase between buoyant prelysosomal vacuoles and dense, acid hydrolase-rich lysosomes in various mammalian cells (Fig. 5).

All of these data are consistent with the evolution in eukaryotes of a diverse family of acidic vacuoles that mediate between primary endosomes and recipient lysosomes. There are specialized forms; e.g., the acidosomes in *Paramecium*, the receiving vacuoles in renal tubular epithelium, and the CURL, which processes internalized receptor/ligand complexes. However, all have in common at least the job of sorting internalized plasma membrane constituents and cargo. In addition, they may be the repository of H^+-ATPase in the endocytic circuit. Despite their intermittent merger with primary endosomes, on the one hand, and digestive compartments, on the other, these vacuoles appear to constitute a discrete organelle, stable but not static.

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