ENDOCYTOSIS IN *ENTAMOEBA HISTOLYTICA*

Evidence for a Unique Non-acidified Compartment

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Endocytosis by mammalian cells leads to the rapid uptake of exogenous solutes within plasma-membrane–derived vesicles. In general, fusion of these vesicles with primary or secondary lysosomes occurs and most biological macromolecules are degraded to small molecular weight components (1). Extensive bidirectional flow of membrane takes place between the plasma membrane and the lysosomal apparatus during endocytosis (7). Recent evidence has suggested that solutes pass through at least one intracellular compartment before reaching the lysosome (2–6) and representative portions of endocytic markers (2, 4, 6, 8) are returned to the cell surface.

We now report the existence of a distinct, intermediate vacuolar compartment in the parasitic amoeba, *Entamoeba histolytica.* This is the primary pinocytic route in this organism. It is equilibrated with the pH of the ambient milieu and allows for the flow of solutes into and out of the cytoplasm. Only after many hours of intracellular residence are the solutes detected in a separate, acidified lysosome-like compartment.

Materials and Methods

Culture of E. histolytica. *E. histolytica* trophozoites, strain HM1:IMSS, were generously provided by Dr. L. Diamond, the National Institute for Allergy and Infectious Diseases, and were maintained in axenic culture in TYI-S medium (9). For studies on internalization of fluid phase markers, fluorescein-labeled dextran (fluorescein isothiocyanate [FITC][1]-dextran, average molecular weight 70,000, Sigma Chemical Co., St. Louis, MO) or horseradish peroxidase (HRP, Sigma) was added directly to the culture medium at concentrations ranging between 1 and 10 mg/ml. After incubation for the indicated times, amoebae were harvested by chilling followed by centrifugation at 500 *g* for 5 min. Trophozoites were then washed by resuspension in cold 19 mM phosphate buffer, pH 7.2, containing 0.27 mM NaCl (PD), or in TYI-S medium and centrifugation. For studies on reincubation of amoebae, trophozoites were harvested and washed with TYI-S medium, resuspended in TYI-S medium, and gently spun (50 *g*) onto the sides of culture tubes. The cultures were then carefully transferred to a 37°C water bath and incubated on their sides. These manipulations were necessary to insure rapid warming of amoebae on reincubation.

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; PD, cold 19 mM phosphate buffer, pH 7.2, containing 0.27 mM NaCl; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA; trichloroacetic acid; TYI-S.

 J. EXP. MED. © The Rockefeller University Press · 0022-1007/84/09/0724/14 $1.00
Volume 160 September 1984 724–737

FITC-Dextran Quantitation and Localization. Samples of culture medium or washed trophozoites containing FITC-dextran were diluted into PD containing 0.1% of the nonionic detergent, Nonidet NP-40 (NP-40, Particle Data Laboratories, Elmhurst, IL). Quantitation of FITC-dextran in solution, pH 7.5, was determined by fluorescence emission at 520 nm in a Perkin-Elmer fluorescence spectrophotometer with the excitation wavelength set at 485 nm and compared to standards of known concentrations.

For the determination of cell-associated fluorescence, trophozoites were harvested and washed twice in cold PD. The concentrated trophozoites were viewed directly in a Zeiss photomicroscope III equipped with fluorescence optics. For photomicroscopy, trophozoites were fixed in PD containing 1% formalin for 30 min at room temperature in the dark.

Determination of Intracellular pH. The pH of the intracellular FITC-dextran environment was determined by the method of Ohkuma and Poole (10). Trophozoites were allowed to attach to coverslips. The coverslips were mounted in a specially designed teflon holder (10), generously provided by the late Dr. Poole of The Rockefeller University, and maintained in a thermostatted cuvette holder. The fluorescence emission at 520 nm was recorded while the excitation wavelength was varied from 400 to 500 nm. Background emission spectra were subtracted and the ratio of fluorescence at excitation wavelengths of 485 nm and 450 nm was determined. A standard curve of fluorescence ratios vs. pH of TYI-S culture medium was constructed and was found to agree with published standards in a variety of simple buffers (10).

HRP Quantitation and Cytochemistry. HRP activity was determined using o-dianisidine as the hydrogen acceptor (11). 1 U of activity resulted in the decomposition of 1 μmol of H_2O_2/min at 25°C. Samples of medium or amoebae containing HRP (specific activity = 440 U/mg) were diluted or solubilized in 10 mM Tris, pH 7.2, containing 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and 0.1% NP-40. To determine the localization of intracellular HRP, trophozoites were incubated with 10 mg/ml of HRP for 4 h, harvested and washed twice with PD, and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 10 min at room temperature. The trophozoites, equilibrated with 10 mM Tris buffer, pH 7.2, for 60 min, were stained for peroxidase activity using diaminobenzidine (12). Trophozoites were washed with 0.9% NaCl, then fixed for 60 min on ice with 1% osmium tetroxide and 40 min on ice with 0.1% uranyl acetate. Fixed trophozoites were pelleted in 2% agarose, dehydrated through a graded series of alcohol, and embedded in Epon. This sections (1,000 Å) were viewed in a Siemens Elmiskop I electron microscope.

Iodination and Isolation of the Surface Membrane. Externally disposed surface peptides of *E. histolytica* were radiolabeled with ^{125}I by the lactoperoxidase and glucose oxidase procedure as previously described (13, 14). Protein-associated ^{125}I was determined by trichloroacetic acid (TCA) precipitation of total amoebal proteins or proteins in subcellular fractions and quantitated using a Packard 5220 gamma counter. Peptides were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (15) after solubilization in SDS. The radiolabeled peptides were visualized by autoradiography on XOMAT XAR-5 (Kodak, Rochester, NY).

Plasma and internal membranes of *E. histolytica* were isolated following treatment of intact amoebae with concanavalin A (Con A), but without the α-methyl mannoside release step (13). The Con A bound to the surface of the trophozoite both modifies the density of the plasma membrane and helps to prevent its fragmentation during isolation (13). Briefly, amoebae were chilled to inhibit endocytosis and were incubated with 0.5 mg/ml of Con A for 5 min on ice. Trophozoites were then swollen in 10 mM Tris buffer, pH 7.2, containing PMSF and 1 mM Mg^{++} for 10 min on ice and homogenized in a Dounce homogenizer. The homogenate was layered over a discontinuous gradient of 0.5 M mannitol, 20% sucrose, and 60% sucrose, all in 10 mM Tris buffer, and spun for 30 min at 250 *g*. Plasma membranes, with the associated Con A, were recovered from the interface between 20% and 60% sucrose. Soluble proteins and internal membranes remained above the mannitol phase. This latter fraction was spun at 48,000 *g* for 1 h to concentrate the internal membranes, which were used for analysis of ^{125}I labeled peptides.

Acridine Orange Staining. Acridine orange (Sigma) was used as a vital stain for lysosomes (16, 17). Trophozoites were incubated for 30 min in TYI-S medium containing 5 μg/ml acridine orange. Amoebae were then washed and viewed by fluorescence microscopy in the living state. Photomicrography was performed through a red filter to reduce background fluorescence.

Results

Fluid Phase Pinocytosis in E. histolytica. HRP and FITC-dextran uptake were used to examine the uptake and distribution of solutes that did not interact strongly with the plasma membrane. Uptake of both markers was temperature dependent and no cell-associated fluorescence or enzymatic activity was detected in cells incubated at 4°C. At 37°C, solute uptake was proportional to the initial concentration of the marker, indicating that these compounds behave as true fluid phase markers for trophozoites of *E. histolytica.*

Fig. 1 shows that the internalization of either 1 mg/ml FITC-dextran or 1 mg/ml HRP by trophozoites was rapid and was linear for 60 min at 37°C. Beyond this time, the rate of uptake slowed, reaching a steady state value at 2 h. When the concentration of FITC-dextran or HRP was increased 10-fold to 10 mg/ml, internalization occurred with the same time course as before; however, the amount of internalized markers was increased 10-fold. In each case, the amount of marker endocytosed corresponded to ~5 μl of fluid taken up by 10^6 amoebae, or ~15% of the total cell volume at equilibrium.

Localization of Pinocytic Markers. FITC-dextran and HRP was visualized in cells by fluorescence microscopy and cytochemical staining, respectively. The photomicrograph in Fig. 2 shows that the fluorescence in trophozoites incubated in FITC-dextran was contained in large intracellular vacuoles >2 μm diameter and some smaller vesicles (<2 μm diameter). Cytochemical demonstration of HRP further defined the intracellular localization of endocytosed fluid phase markers. Nearly all of the large vacuoles and a proportion of small vesicles (<2 μm diameter) contained reaction product in HRP-treated amoebae (Fig. 3*A*). Vacuoles and vesicles of trophozoites not incubated with HRP were devoid of reaction product (Fig. 3*B*).

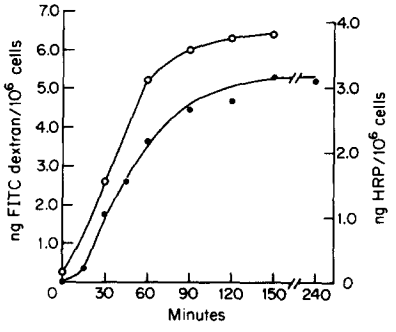

FIGURE 1. Uptake of fluid phase markers by *E. histolytica.* Trophozoites were incubated in either 1 mg/ml FITC-dextran (●) or 1 mg/ml HRP (○) for the indicated times. After removal of the extracellular marker by washing twice with cold PD, the cells were solubilized in 0.1% Nonidet NP-40 in PBS, pH 7.5. FICT-dextran was determined in cell extracts by fluorescence with the excitation wavelength at 485 nm and the emission wavelength set at 520 nm. Cell-associated HRP was measured spectrophotometrically as peroxidase activity.

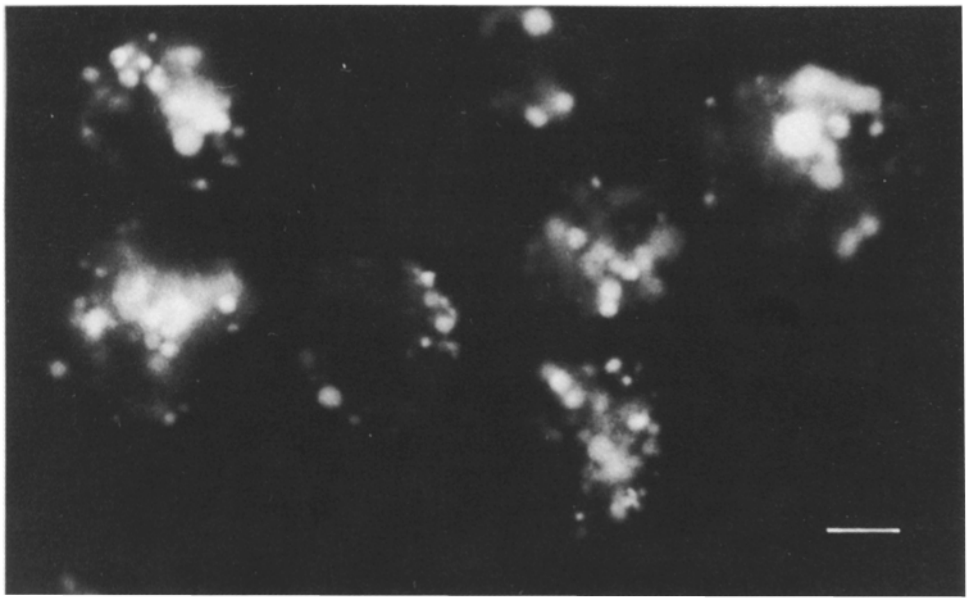

FIGURE 2. Intracellular localization of FITC-dextran in *E. histolytica* as visualized by fluorescence microscopy. Trophozoites were incubated in 1 mg/ml FITC-dextran for 3 h, then washed with PD and fixed in PD containing 1% formalin for 30 min at room temperature in the dark. Bar, 10 μm. × 960.

Fate of Endocytosed Markers. The reduction in solute uptake was not due to a cessation of endocytosis as shown in the following experiment. Trophozoites were incubated in a medium containing FITC-dextran (1 mg/ml) until uptake reached a plateau at 2 h (Fig. 4). At this time, HRP was added to the medium to a final concentration of 1 mg/ml. The trophozoites proceeded to take up HRP with normal kinetics for 90 min while maintaining the steady state level of FITC-dextran (Fig. 4). An equilibrium had therefore been reached between uptake and destruction or efflux of the fluid phase markers.

The relative importance of degradation and efflux was followed in a series of washout experiments. Trophozoites were loaded with 1 or 10 mg/ml FITC-dextran until the steady state was established (4 h) and then returned to culture in the absence of the marker. Rapid reduction in cell-associated fluorescence occurred over a period of 90 min (Fig. 5) with a concomitant increase in fluorescence in the culture medium. Sephadex G-200 chromatography indicated that the FITC-dextran was released intact into the medium. Native FITC-dextran in TYI-S medium (Fig. 6*A*) had a chromatographic profile similar to that of fluorescent material recovered from the washout medium (Fig. 6*B*) or from washed cells following detergent lysis (Fig. 6*C*). TYI-S culture medium contained some fluorescent material (Fig. 6*D*), which accounted for the low molecular weight fluorescent product in the washout medium. At no stage of the incubation was there significant degradation of the FITC-dextran backbone, indicating that the intact molecule was being taken up and released by trophozoites in a continuous fashion. The rapid phase of uptake was presumably the time necessary to fill the large vacuole compartment.

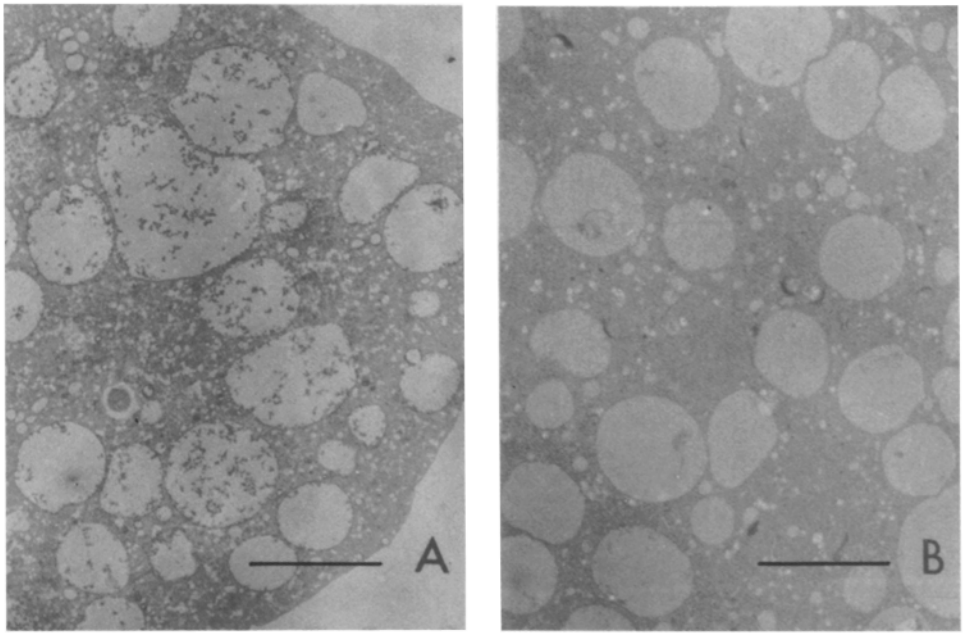

FIGURE 3. Intracellular localization of HRP in *E. histolytica* as visualized by electron microscopy. Trophozoites were incubated with or without 10 mg/ml HRP for 6 h, and stained for peroxidase activity. (*A*) HRP. Note the localization of reaction product within the large vacuoles. (*B*) No HRP. Bar, 5 μm. × 3,430.

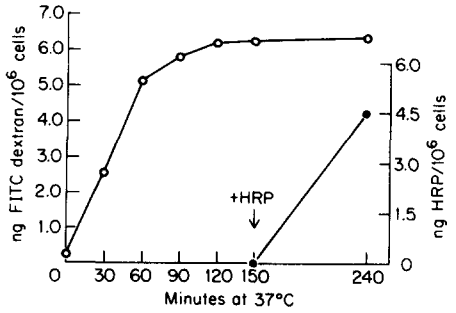

FIGURE 4. HRP uptake by *E. histolytica* trophozoites containing steady-state concentrations of FITC-dextran. (○) Cells were incubated in 1 mg/ml FITC-dextran and the cell-associated fluorescence was determined as in Fig. 1. (●) At 150 min, 1 mg/ml HRP was added to the culture medium, and cell-associated peroxidase activity was determined at 150 and 240 min.

The efflux of pinocytic marker by *E. histolytica* was also demonstrated using HRP. When amoebae incubated with 5 mg/ml HRP were returned to culture in fresh medium, peroxidase activity was progressively lost from the cells and up to 100% of the activity could be recovered in the culture medium (Fig. 7).

pH of Endocytic Vacuoles. The fluorescence spectrum of FITC-dextran is an indicator of pH in intracellular compartments (10). When trophozoites were incubated in 10 mg/ml FITC-dextran for 3 h, the pH of vacuoles with the marker was found to be 6.3 ± 0.2 (Table I), which was only slightly more acid than the pH (6.8) of the culture medium.

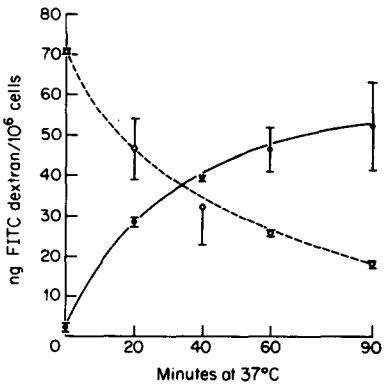

FIGURE 5. Release of intracellular FITC-dextran by *E. histolytica* trophozoites and recovery in the culture medium. Trophozoites were incubated in 10 mg/ml FITC-dextran for 3 h, then washed, and returned to culture in fresh medium. (○) Cell-associated FITC-dextran determined as described in Fig. 1. (●) FITC-dextran recovered from the culture medium.

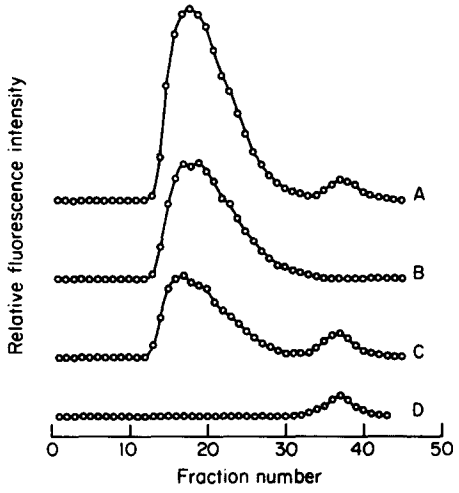

FIGURE 6. Absence of FITC-dextran degradation by *E. histolytica*. Trophozoites were incubated for 4 h with 10 mg/ml FITC-dextran and harvested. The media were saved and the cells were washed twice with cold PD. The cells were either reincubated in fresh TYI-S medium or solubilized in PD plus 0.1% Nonidet NP-40. Cell lysates and medium were chromatogaphed on an 1.4 × 80 cm Sephadex G-200 column. FITC-dextran in column eluates was determined by fluorescence as described in Fig. 1. (*A*) FITC-dextran dissolved in fresh culture medium. (*B*) Trophozoites incubated (4 h) with FITC-dextran, then washed free of medium and solubilized in nonionic detergent. (*C*) Medium containing FITC-dextran released from cells after a 90-min incubation in fresh medium. (*D*) TYI-S culture medium showing low molecular weight fluorescent material inherent in the medium.

Trophozoites were also cultured in TYI-S medium plus FITC-dextran, adjusted to pH 7.5 or 8.0, for 3 h and the pH of the endocytic vacuoles determined. The pH of the endocytic vacuoles was found to vary with the pH of the medium. Vacuolar pH values of 7.5 ± 0.5 and >8.0 were measured for trophozoites in media of pH 7.5 and 8.0, respectively (Table I, lines 1–3).

Acridine Orange Staining. The above data indicated that the vacuoles of *E. histolytica* were not acidified and that their pH was determined by that of the

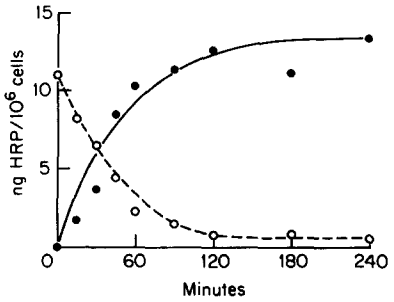

FIGURE 7. Release and recovery of HRP by *E. histolytica*. Trophozoites were incubated in 5 mg/ml HRP for 3 h, then washed and returned to culture in fresh medium. (○) Cell-associated peroxidase activity determined after various times of incubation. (●) Total peroxidase activity recovered from the medium after various times of incubation.

TABLE I

Effect of Exposure Time to FITC-Dextran and pH of the Medium on the pH of E. histolytica Endocytic Vesicles

Sample	pH of vesicle*	pH of medium
1. FITC-dextran, 3 h	6.3 ± 0.2	6.8
2. FITC-dextran, 3 h	7.7 ± 0.3	7.5
3. FITC-dextran, 3 h	≥8.0	8.0
4. FITC-dextran, 3 d 10-h chase	5.1 ± 0.4	6.8
5. FITC-dextran, 3 h 30-min chase	6.2 ± 0.4	6.8

E. histolytica trophozoites were incubated in TYI-S medium containig 10 mg/ml FITC-dextran for the stated times. The cells were washed with warm medium and allowed to attach to glass coverslips that were mounted into a cuvette holder for determination of fluorescence spectrum. pH values were established by comparison with a standard curve obtained by measurement of FITC-dextran fluorescence in solutions of known pH. Where indicated, trophozoites were washed and transferred to fresh medium without FITC-dextran for the chase period before determination of pH values.

* Vesicle pH is the mean ± standard deviation of three determinations.

medium. This is in contrast to most cell types where endocytosis is primarily directed to move material into the lysosomal compartment of the cell for digestion. For this reason, acridine orange, a metachromatic vital dye, was used to assay directly for an acidified vesicle compartment in *E. histolytica*. Mammalian cells concentrate the dye within lysosomes that fluoresce red-orange on a yellow background (16). When trophozoites were cultured in acridine orange, numerous small vesicles (<2 μm) were labeled (Fig. 8). All other areas of the cell, including the large vacuoles labeled by endocytic markers, did not fluoresce. Thus, an acidified vesicle compartment is evident in *E. histolytica*.

Identification and pH of Secondary Lysosomes. The nature of the acridine orange–staining compartment and its association with endocytic vesicles was further explored. It was found that labeling of the small vesicle compartment also could be achieved after prolonged exposure of trophozoites to FITC-dextran. We therefore designed experiments in which dextran was concentrated in these slowly exchanging vesicles and was washed out of the larger vacuoles.

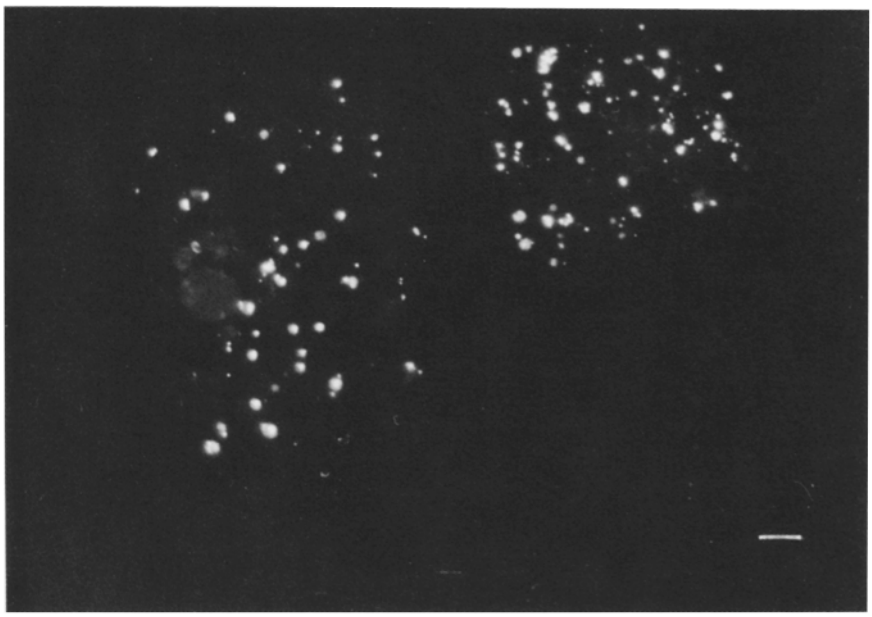

FIGURE 8. Acridine orange staining of lysosomes of *E. histolytica*. Trophozoites were incubated for 30 min in TYI-S medium containing 5 μg/ml acridine orange, washed with PD, and viewed by fluorescence microscopy as viable preparations. Bar, 10 μm. × 560.

For this purpose, trophozoites were loaded with FITC-dextran (10 mg/ml) for 3 d and then incubated in fresh medium (no marker) for 10 h. These cells, when viewed in the fluorescence microscope, demonstrated the labeling of numerous smaller vesicles (Fig. 9) in contrast to the larger vacuoles with shorter pulses (see Fig. 2). The fluorescence spectrum of the smaller vesicles indicated that their pH was 5.2, considerably below that of the external environment (Table I, line 4), and was consistent with a lysosomal compartment. As noted above, this is in contrast to the lack of acidification of vacuoles after a short (3 h) exposure to dextran followed by a 30-min incubation in fresh medium (Table I, line 5) to reduce background fluorescence.

Internalization of Plasma Membrane. Prior studies reported the isolation of a highly enriched plasma membrane from *E. histolytica* as well as an associated group of internal vesicles (13). These latter vesicles may represent a mixture of both the rapidly and slowly exchanging endocytic compartments detected in the above experiments. To examine the rate of plasma membrane internalization, the externally disposed peptides on the amoebal surface were labeled at 0°C with ^{125}I using the lactoperoxidase-glucose oxidase procedure. Immediately thereafter and at selected intervals at 37°C or 0°C, the amoebae were homogenized and the plasma membrane and internal vesicles isolated (Materials and Methods). When ^{125}I-labeled trophozoites were incubated on ice, 85% of the radiolabel was recovered in the plasma membrane fraction. However, as shown in Fig. 10, there was a progressive increase in the labeled membrane peptides of the internal vesicle compartment at 37°C over a 2-h period until 60% of the label had been interiorized. The specific activity of the radiolabel in protein

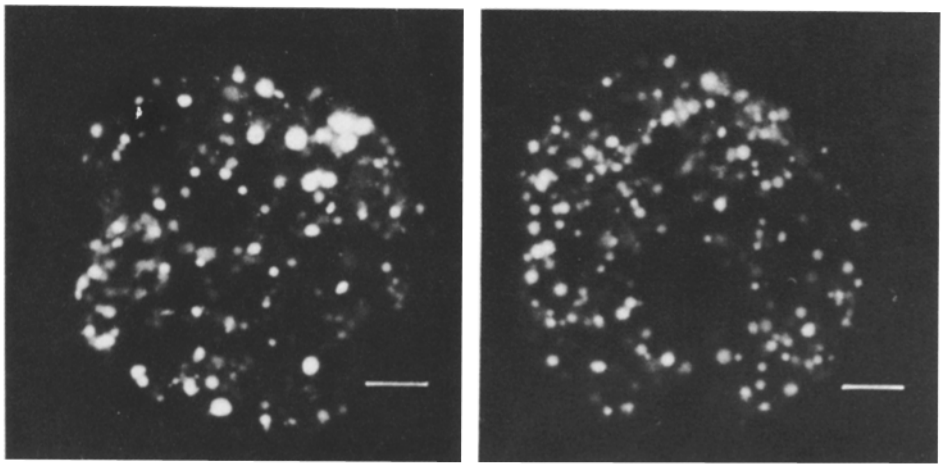

FIGURE 9. Distribution of residual FITC-dextran in *E. histolytica* as determined by fluorescence microscopy. Trophozoites were incubated in 10 mg/ml FITC-dextran for 3 d, washed, and incubated for 10 h in fresh medium to eliminate marker from the rapidly exchanging vacuolar pool. Cells were washed, fixed, and viewed as described in Fig. 3. Residual FITC-dextran appeared to be mostly confined to the small vesicles (<2 μm diameter) of the cell. Bar, 10 μm. × 800.

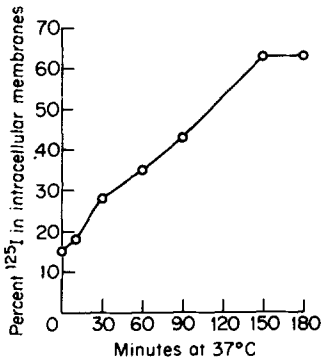

FIGURE 10. Redistribution of ^{125}I surface radiolabel on trophozoites of *E. histolytica*. Intact trophozoites were radiolabeled with ^{125}I and returned to culture in fresh medium. At various times, the cells were harvested and the subcellular membranes separated into surface membrane and internal membranes. TCA-precipitable radiolabel was determined for each membrane fraction.

varied only slightly over a 4-h period, as determined from the amounts of TCA-precipitable ^{125}I and total cell protein. Therefore, degradation of surface peptides did not occur at a sufficiently rapid rate to account for the appearance of radiolabel in internal cell membranes. Furthermore, SDS gel electrophoresis of the internal vesicle fraction revealed that the majority of polypeptides have similar molecular weights as those initially iodinated on the cell surface (Fig. 11), the exception being the absence of a major set of bands in the 40,000-molecular weight range in the vesicle fraction. These observations taken together suggested that representative samples of the amoebal plasma membrane had been interiorized as endocytic vesicles. In view of the rapid time course, it is likely that this

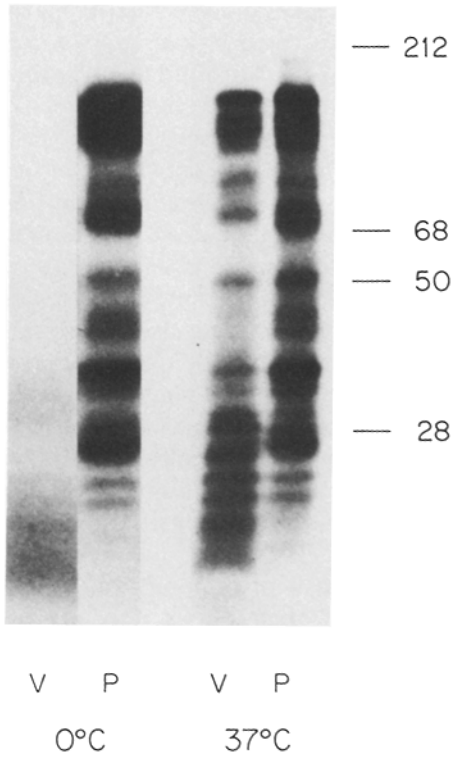

FIGURE 11. Redistribution of surface ^{125}I radiolabeled plasma membrane peptides of *E. histolytica*. Trophozoites were radiolabeled and incubated in culture medium at either 4°C or 37°C for 30 min. Surface and interior membranes were isolated and the membrane peptides separated by SDS-PAGE after solubilization in SDS. (*P*) Surface membrane. (*V*) Internal membranes.

redistribution represented communication of the plasma membrane with the rapidly exchanging large vacuole fraction.

Discussion

By electron microscopy *E. histolytica* trophozoites are devoid of a number of common intracellular membrane organelles (18, 19). There are no recognizable primary lysosomes, Golgi apparatus, membrane-bound ribosomes, or organized endoplasmic reticulum. Instead, the amoebae are filled with large numbers of membrane-bound structures, which can be loosely divided into large vacuoles (>2 μm) and small vesicles (<2 μm). Previously, we have shown that the internal membranes and plasma membrane of the amoebae can be isolated separately and that a number of markers, including a membrane-bound acid phosphatase, are shared between the two membrane classes (13).

It is now clear that extensive and rapid communication exists between the cell surface and the internal vesicles of *E. histolytica*. Fluid phase markers such as fluoresceinated dextran and HRP are accumulated intracellularly, equilibrating within the contents of the large vacuolar compartment within 1–2 h. This equilibrium can be maintained for many hours in culture and is apparently a

balance between the rate of uptake and the rate of exocytosis. The extent and rate of solute efflux was demonstrated by pulse-chase experiments in which the intracellular markers declined to background levels with a half-life of ~40 min.

In mammalian systems, the uptake of pinocytic markers remains linear or nearly linear for considerable periods. Sensitive assays have shown that once macromolecules are delivered to lysosomes (1), little or no exocytosis occurs. The major portion of exogenous molecules are either digested or retained for longer periods (20) within lysosomes of most well-studied mammalian systems. For example, in lysosomes of mammalian phagocytes, the half-life of the bulk of HRP is ~8 h and <1% escapes into the medium in macromolecular form. On the other hand, since membrane recycling occurs during endocytosis (1), some internalized fluid should be returned to the extracellular space. Besterman et al. (4) showed that [^{3}H]sucrose is released by macrophages and fibroblasts, possibly from the pinocytic compartment. Likewise, Adams et al. (2) found release of the macromolecular tracer HRP from CHO cells, again from a pinocytic compartment. In these cases, the extent of marker loss (≥30%) from this pool is less than the quantitative loss of HRP and fluoresceinated dextran exhibited by *E. histolytica.*

The large vacuole compartment of *E. histolytica*, which is associated with the rapid influx and efflux of exogenous solutes, does not have the properties of a secondary lysosome. First, the contents of these organelles are not acidified, reflecting instead the hydrogen ion concentration of the external environment. Second, no appreciable degradation of the solutes occurred during their 2- or 3-h transit through the non-acidified vacuolar compartment. We could find no evidence for an intermediate, acidified compartment described in fibroblasts (3). Only after prolonged (days) incubation could fluorescinated dextran be detected in the acidified small vesicle compartment of *E. histolytica.* It is not clear at this time whether the solute is transferred from one intracellular membrane compartment to another or whether degradation of the solute occurs. Transfer could be accomplished by a slow conversion of the endocytic vacuolar compartment involving a gradual shrinkage in vacuole size or a budding off of small vesicles. These vesicles would be subsequently acidified, possibly by fusion with lysosomes. Our observation that these cells contain vesicles that accumulate acridine orange provides indirect evidence for the existence of lysosomes. However, discrete enzymatic markers for *E. histolytica* lysosomes are lacking, thus precluding their isolation and characterization. An acid phosphatase has been described in this organism (13, 21), but is a membrane-associated activity localized both on surface and internal membranes (13).

These results differ from those of Heiple and Taylor (22) who measured pH changes in pinosomes and phagosomes of *Chaos carolinesis* by microfluorometric techniques. Acidification began within 5 min of phagosome formation and was complete by 20 min. Pinosomes were also found acidified, but pinocytosis was induced in acidified medium. For this reason, it remains to be determined whether fusion with lysosomes is an early event in the pinocytic cycle of *C. carolinesis* or whether the pH of pinosomes reflects that of the external environment.

Within the protozoa, the exocytosis of insoluble residues of phagocytosis is a

well-known phenomenon (23). This process usually requires much longer transit times than we have shown for *E. histolytica*, and is generally an organized pathway in which defecation occurs only after the contents of the endocytic compartment are fully digested. However, the release of only a portion of the contents of a vacuole has been reported for the reptile parasite *Entamoeba invadens* (24). Prusch (25) reported the bulk extrusion of dye by *Amoeba porteus* while the concentration of sucrose remained constant, suggesting that discriminate fates are possible for solutes taken up by pinocytosis.

In addition to endocytosis of solutes, *E. histolytica* trophozoites readily ingest and break down particles such as human erythrocytes (26). Little is known concerning the time course of such degradation or of the extent of digestion of the particles, especially of their soluble components. It would be interesting to know whether phagocytosed particles must pass through the same or similar non-degradative compartment, or whether the fate of an endocytic vesicle is determined by its contents.

Summary

Our studies on endocytosis in *Entamoeba histolytica* trophozoites suggest that there are two vacuolar compartments in this organism. The first compartment consists of large vacuoles (>2 μm diameter). As measured by the fluid phase markers, fluorescein isothiocyanate (FITC)-dextran and horseradish peroxidase (HRP), this compartment is a rapid equilibrium with the external milieu and is constantly exchanging (1–2 h) its contents with the external medium. The contents of these vacuoles are not acidified. This together with the absence of degradation of fluid phase markers clearly differentiates these vacuoles from lysosomes of eucaryotes. By labeling externally disposed peptides on the surface membrane of trophozoites with ^{125}I, we could show that the surface membrane was rapidly internalized over a 2-h period and then reached a plateau. All major ^{125}I surface proteins, with the exception of a set of peptides in the 40,000 molecular weight range, were interiorized and ~60% of the total radiolabel were found to be in the internal membrane fraction at any given time. The kinetics of this process were similar to those for the uptake of fluid phase markers and are best explained by cycling of the surface membrane into the vacuolar compartment(s) and then back to the cell surface.

The second vacuolar compartment consisted of small vesicles (<2 μm diameter) with acidified contents as indicated by acridine orange uptake. The endocytic nature of these vesicles was shown by their slow (days) labeling with FITC-dextran, and spectral analysis of internalized FITC-dextran confirmed that this second compartment is acidified (pH 5.2).

Received for publication 14 March 1984 and in revised form 24 May 1984.

References

1. Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* 96:1.
2. Adams, C. J., K. M. Maurey, and B. Storrie. 1982. Exocytosis of pinocytic contents by hamster ovary cells. *J. Cell Biol.* 93:632.

3. Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing α_2-macroglobulin. *Cell.* 28:643.
4. Besterman, J. M., J. A. Airhart, R. C. Woodworth, and R. B. Low. 1981. Exocytosis of pinocytosed fluid in cultured cells: kinetic evidence for rapid turnover and compartmentation. *J. Cell Biol.* 91:716.
5. Wall, D. A., and A. L. Hubbard. 1981. Galactose-specific recognition system of mammalian liver: receptor distribution on the hepatocyte cell surface. *J. Cell Biol.* 90:687.
6. Connolly, D. T., R. R. Townsend, R. Kawaguchi, W. R. Bell, and Y. C. Lee. 1982. Binding and endocytosis of cluster glycosides by rabbit hepatocytes. Evidence for a short-circuit pathway that does not lead to degradation. *J. Biol. Chem.* 257:939.
7. Muller, W. A., R. M. Steinman, and Z. A. Cohn. 1980. The membrane proteins of the vacuolar system. II. Bidirectional flow between secondary lysosomes and the plasma membrane. *J. Cell Biol.* 86:304.
8. Berlin, R. D., J. P. Fera, and J. P. Pfeiffer. 1979. Reversible phagocytosis in rabbit polymorphonuclear leukocytes. *J. Clin. Invest.* 63:1137.
9. Diamond, L. S., D. R. Harlow, and C. C. Cunnick. 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other Entamoeba. *Trans. R. Soc. Trop. Med. Hyg.* 72:431.
10. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327.
11. Worthington Enzyme Manual. 1977. Worthington Biochemical Corp. Freehold, NJ. p. 66.
12. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of adsorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291.
13. Aley, S. B., W. A. Scott, and Z. A. Cohn. 1980. Plasma membrane of *Entamoeba histolytica. J. Exp. Med.* 152:391.
14. Hubbard, A. L., and Z. A. Cohn. 1972. The enzymatic iodination of the red cell membrane. *J. Cell Biol.* 55:390.
15. Maizel, J. V., Jr. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* 5:179.
16. Robbins, E., and P. I. Marcus. 1963. Dynamics of acridine orange-cell interaction. I. Interrelationships of the acridine orange particles and cytoplasmic reddening. *J. Cell Biol.* 18:237.
17. de Duve, C., T. de Barsy, B. Poole, A. Tronet, P. Tulkens, and F. van Hoof. 1974. Lysosomotropic agents. *Biochem. Pharmacol.* 23:2495.
18. Lowe, C. Y., and B. C. Maegraith. 1970. Electron microscopy of an axenic strain of *Entamoeba histolytica. Ann. Trop. Med. Parasitol.* 64:293.
19. Proctor, E. M., and M. A. Gregory. 1972. The ultrastructure of axenically cultivated trophozoites of *Entamoeba histolytica* with particular reference to an observed variation in structural pattern. *Ann. Trop. Med. Parasitol.* 66:335.
20. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase (HRP)-anti-HRP immune complexes with mouse peritoneal macrophages *in vitro. J. Cell Biol.* 55:616.
21. McLaughlin, J., D. G. Lindmark, and M. Muller. 1978. Inorganic pyrophosphatase and nucleoside diphosphatase in the parasitic protozoon, *Entamoeba histolytica. Biochem. Biophys. Res. Commun.* 82:913.
22. Heiple, J. M., and D. L. Taylor. 1982. pH changes in pinosomes and phagosomes in the ameba, *Chaos carolinensis. J. Cell Biol.* 94:143.

23. Chapman-Andresen, C. 1973. Endocytic processes. *In* The Biology of Amoeba. K. W. Jeon, editor. Academic Press, New York. pp. 319–348.
24. Zaman, V. 1962. An electron microscopic observation of starch ingestion by *Entamoeba invadens. Trans. R. Soc. Trop. Med. Hyg.* 56:535.
25. Prusch, P. D. 1981. Bulk solute extrusion as a mechanism conferring solute uptake specificity by pinocytosis in *Amoeba proteus. Science (Wash. DC).* 213:668.
26. Trissl, D., A. Martinez-Palomo, C. Arguello, M. de la Torre, and R. de la Hoz. 1977. Surface properties related to concanavalin A-induced agglutination. A comparative study of several Entamoeba Strains. *J. Exp. Med.* 145:652.