IN VITRO FUSION OF *ACANTHAMOEBA* PHAGOLYSOSOMES

I. Demonstration and Quantitation of Vacuole Fusion in *Acanthamoeba* Homogenates

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ABSTRACT

Fusion of phagolysosomes (PLs) has been demonstrated to occur in vitro. Two separate cell homogenates of the ameba *Acanthamoeba* sp. (Neff) were prepared, each rich in PLs labeled with distinctive particulate markers. Portions of each were incubated together in vitro and fusion occurred as evidenced by the appearance of PLs containing both types of markers. Fusion was confirmed by electron microscopy, including serial sectioning. The membranes of fused vacuoles excluded the dye eosin Y. Surviving cells in the homogenates were not responsible for the observed fusion. Fusion was obtained using either synthetic markers (polystyrene and polyvinyltoluene latex) or biological markers (autoclaved yeast cells and glutaraldehyde-fixed goat red blood cells), or a combination of both. The specificity of PL fusion in vivo appeared to be maintained in vitro.

As determined by light and electron microscopy, the fusion reaction was dependent on time and temperature, and on the initial presence of membrane around both marker particles. A minimum of 10% of the vacuoles fused by 10 min of incubation at 30°C, and no rupture of the vacuoles was detected during this time. After 10 min of incubation, vacuole rupture began and fusion ceased. At a constant initial vacuole concentration, the extent of PL fusion in vitro was quantitatively reproducible. This appears to be a promising system for further investigation of membrane fusion in the lysosomal system.

Lysosomes are seemingly ubiquitous cellular organelles, occurring in most eukaryotic cell types including virtually all mammalian cells so far examined (20). According to our current understanding, the functioning of the lysosomal system relies on one central ultrastructural mechanism: membrane fusion of cytoplasmic vacuoles (5, 6). Experimentally, very little is known about the factors involved in the fusion of vacuolar membranes. In vivo studies have been able to demonstrate the existence of vacuole fusion (3), but the lack of an in vitro experimental system has precluded any straightforward biochemical study of vacuole fusion and has severely limited progress in our understanding of this process. Evidence suggesting that isolated lysosomes might be capable of spontaneous fusion has recently been described in a brief note (25).

We report here the development of an in vitro system in which the fusion of vacuoles (phagolysosomes)[1] occurs. The reaction occurs

[1] In this report the term "vacuole" is used to refer to the combined phagosome-phagolysosome population, which consists predominantly of phagolysosomes (see Discussion).

rapidly, in substantial yield, with high specificity, and under conditions reasonably close to those existing in the cell. With this system, it has proved possible to obtain quantitative data concerning the fusion reaction. This system should therefore permit the defining of some of the biochemical and biophysical factors involved in this process, at least as it occurs in vitro. To our knowledge, the present report is the first description of such a system.

The cell type used for these studies was *Acanthamoeba* sp. (Neff), a small, free-living ameba which can be grown axenically in mass culture (19) and which shows a high capacity for the endocytosis of particulate substances (24). Phagocytosed particles appear to interact rapidly with the lysosomal system of this organism, as many of the phagosomes are converted into phagolysosomes soon after ingestion (see Discussion). The experimental approach used to demonstrate in vitro fusion was as follows: two separate populations of *Acanthamoeba* phagolysosomes were prepared, each containing a distinct type of particulate marker (e.g., yeast cells or glutaraldehyde-fixed goat red blood cells [GGRBC]).[2] The two populations were mixed and incubated together in vitro, and fusion was detected by light and electron microscopy, i.e. by the detection of vacuoles which contained both markers (hybrids). An abstract of this work has been previously published (21).

MATERIALS AND METHODS

Materials

Monodisperse polystyrene (PS) and polyvinyltoluene (PVT) latex spheres were purchased from Diagnostic Products. Indianapolis, Ind. Stated diameter distributions were 0.807 ± 0.005 μm (PS) and 2.02 ± 0.01 μm (PVT). *Saccharomyces cerevisiae* (Anheuser-Busch strain) were kindly provided by Dr. R. J. Neff of this department. Whole goat blood (1:1 in Alsever's solution) was purchased from GIBCO (Grand Island Biological Co.) Diagnostics, Madison, Wis. A Cenco stroboscope (model 74675, Cenco Instruments Corp., Chicago, Ill.) was used to calibrate the model 1 International centrifuge (International Equipment Co., Needham Heights, Mass.). Density gradient grade sucrose and enzyme grade Tris (base) were purchased from the Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. Electron microscopy (EM) grade OsO_4 and glutaraldehyde were obtained from Polysciences, Inc., Warrington, Pa. All other chemicals were reagent grade.

Cell Cultures

Acanthamoeba sp. (Neff) were grown axenically at 30°C in 500-ml aerated cultures as described by Neff et al. (19). Except for early experiments in which the medium was used without treatment, Neff's optimal growth medium (GM) (19) was always used with the following treatment to render it "particle free." 1.5-liter batches were autoclaved for 5 min at 121°C and the resulting precipitate was removed by successively filtering the cooled medium through Whatman no. 40, Whatman no. 42, and Millipore 0.45-μm pore filters (Millipore Corp., Bedford, Mass.). The medium was then reautoclaved for 25–30 min and remained clear. The cells grew normally with a generation time of 8 h. Cell numbers in both ameba and yeast cultures (and in red blood cell preparations) were determined by hemocytometer counts.

Saccharomyces cerevisiae were grown at room temperature (23°C) on 3% glucose-1% yeast extract in 1- or 4-liter aerated cultures, by the same techniques as used for the amebas.

Preparation of Marker Particles

In this procedure and in that following, manipulations were carried out at room temperature and centrifugation steps were done at 5°C. All *g* forces given are those existing at the bottom of the centrifuge tube. All salt solutions used in this work were filtered through 0.45-μm pore Millipore filters before use. Dilutions and resuspensions (e.g., 1:1) were always made on a direct (vol/vol) basis.

AUTOCLAVED YEAST: To facilitate the execution of these experiments, a slowly sedimenting ("light") yeast population was employed (see Discussion). "Light" yeast were prepared as follows. When yeast cultures were dense (about 2×10^8 cells/ml), they were autoclaved for 30–40 min, cooled, and diluted 1:1 with 0.84% NaCl which was 10 mM in potassium phosphate buffer, pH 6.8 (PBS). Four 450-ml portions were centrifuged at 1,000 rpm (250 *g*) for 1.5 min at speed in a model PR-2 International centrifuge (IEC no. 276 rotor). The supernates were carefully aspirated off into a collecting flask and were centrifuged again at 1,000 rpm for 1 min at speed. To sediment the "light" yeast, the supernates from the second step were centrifuged for 25 min at 1,000 rpm. The resulting pellets were combined in a final volume of 40 ml and centrifuged in a 50-ml tube at 2,200 rpm (1,070 *g*; IEC no. 269 rotor) for 10 min. The packed pellet of yeast (1–2 ml) was resuspended 1:1 with PBS, giving a

[2] *Abbreviations used in this paper:* EM, electron microscope; GGRBC, glutaraldehyde-fixed goat red blood cells; GM, growth medium; Gv, GGRBC-containing vacuoles; LM, light microscope; PBS, phosphate-buffered saline; PBSM, phosphate-buffered saline with 1 mM $CaCl_2$ and $MgSO_4$; PLs, phagolysosomes; PS, polystyrene; PVT, polyvinyltoluene; TVp, total vacuole population; TYp, total yeast population; Yv, yeast-containing vacuoles.

stock at approximately 5×10^9 yeast particles/ml, which was stored at 0°C. The yield of "light" yeast particles (2–5 μm in diameter) was 5–10%.

GLUTARALDEHYDE-FIXED GOAT RED BLOOD CELLS: The morphology of the red cells in commercial samples of goat blood varied considerably. The following procedure was developed which reproducibly yielded a uniform spherical population of particles approximately 3.1 μm in diameter. (85–90% spheres, 5–10% ellipsoids, 5–10% ghosts).

41 ml of goat blood (1:1 in Alsever's) was filtered through two layers of gauze. The filtrate was then divided into eight 5-ml aliquots in 50-ml centrifuge tubes and each was diluted to 40 ml with PBS. The cells were pelleted in the model PR-2 International centrifuge at 3,300 rpm (2,400 *g*, no. 269 rotor) for 7 min at speed. The thin layer of white cells was aspirated off along with the supernates. Each pellet was resuspended in PBS (all resuspensions were to 40 ml), and the cells were pelleted again at 3,300 rpm for 5 min. The cells were washed twice more in PBS with centrifugation at 3,000 rpm (2,000 *g*) for 5 min both times. Each pellet was then thoroughly resuspended in 5 ml of PBS with a round-ended Teflon rod or a motor-driven spatula. Each suspension was diluted with 5 ml of 0.80% NaCl, mixed well, and then 25 ml of 2.2% glutaraldehyde (biological grade) was forcefully expelled from a wide-mouthed pipette into each tube. The glutaraldehyde suspensions were pooled in a 500-ml flask and stirred continuously on a magnetic stirrer for 16–20 h at room temperature.

After fixation, the dark maroon suspension was equally divided into eight 50-ml tubes, the volumes were adjusted to 40 ml with PBS, and the tubes were centrifuged at 3,000 rpm for 8 min. The pellets were each washed three times in 40 ml of PBS, with the first two centrifugations at 3,000 rpm for 5 min and the third at 2,800 rpm (1,740 *g*) for 5 min. The cells were then combined into two tubes, resuspended in PBS, and sedimented at 2,100 rpm (975 *g*) for 10 min. The resulting pellets (about 3 ml each) were resuspended 1:3 with PBS, giving GGRBC stocks at approximately 1×10^{10} particles/ml which were stored at 0°C.

In Vivo Fusion Experiments

To a 25-ml culture of *Acanthamoeba* cells at 2×10^6 cells/ml, PS particles (0.8 μm diameter) were added to give 1.4 mg PS/ml (about 2,500 PS particles/cell). After 10 min, the cells were pelleted by low-speed centrifugation, washed three times in 40 ml of cold 0.02 M Tris-HCl buffer, pH 7.4, to remove uningested particles (29), and resuspended in 25 ml of fresh GM at 30°C with aeration. After allowing the cells to "re-equilibrate" for 5 min (9), PVT particles (2 μm diam) were added at 0.4 mg PVT/ml (about 50 particles per cell), and 1-ml samples of the culture were taken at 2, 7, and 10 min after the addition of the PVT particles. The cells in each sample were pelleted by centrifugation, and then fixed and processed at 0°C for EM by the procedure of Wetzel and Korn (29). The course of the experiment was monitored by light microscopy (LM), with samples being taken at frequent intervals.

The same experiment was later performed with GGRBC and yeast particles, the GGRBC being added first since they wash out more easily than the yeast. In this experiment, the washings were done in 30°C GM and the samples were fixed at 30°C by diluting 1:1 with 2% glutaraldehyde which was 0.1 M in potassium phosphate buffer, pH 7.3. The cells were pelleted and fresh fixative was added to the pellets, which were allowed to fix for 2–3 h. Pellets were then washed twice with PBS and postfixed for 2–3 h at 0°C in 1% OsO_4 which was 0.1 M in sodium phosphate buffer, pH 6.9. They were then washed in PBS, dehydrated through ethanol and propylene oxide, and embedded in Epon 812 (15).

In Vitro Fusion Experiments

In the procedures that follow, all solutions (except GM) were prepared with double-distilled deionized water. The Dounce homogenizers (Kontes Glass Co., Vineland, N. J.) and the test tubes used for the in vitro reaction were acid washed. Unless otherwise noted, all steps were carried out at 30°C. In cell cultures used for these experiments, the number of cysts was always less than 2%. An outline of the protocol developed for the in vitro fusion experiments is shown schematically in Fig. 1. The details of each step are as follows.

PHAGOCYTOSIS: From a 500-ml culture of *Acanthamoeba* at $2–3 \times 10^6$ cells/ml, 6×10^8 cells were harvested and divided equally into two 50-ml bubbler tubes in a volume of 30 ml of GM each (1×10^7 cells/ml). Aeration was at 1 cfm. GGRBC particles were added to one culture at a particle to cell ratio of 100:1 (approximately 3 ml of GGRBC stock), and autoclaved "light" yeast were added to the other culture at 50:1 (approximately 3 ml of yeast stock). Particle uptake by the cells and the progress of the rest of the experiment were closely monitored by phase microscopy (low power and oil immersion).

WASHING: When both cultures showed an average ingestion of 20–30 particles/cell (usually after 20–30 min), each culture was divided into four 40-ml centrifuge tubes (8.2 ml/tube) and each portion was diluted to 40 ml with GM. The cells were then gently pelleted in an International centrifuge (model 1) by activating the centrifuge for 1 min, during which time the rotor was accelerated to 1,350 rpm (430 *g*). After the particle-rich supernates were aspirated off, each cell pellet was resuspended to 40 ml with GM and the cells were gently sedimented as above, accelerating to 1,300 rpm (400 *g*) at 1 min, which pelleted 96–98% of the cells. This washing procedure was repeated until both groups of cells were essentially free of uningested particles (free particles/ingested particles $<5\%$). This usually required four more

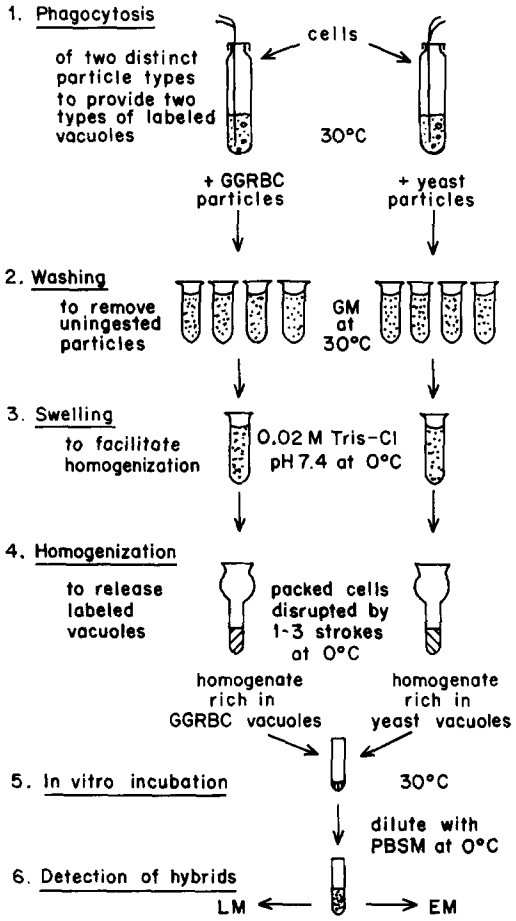


FIGURE 1 Schematic outline of the protocol for the in vitro fusion experiments. The details of each step are given in the text.

washes for the yeast-containing cells and two more for the GGRBC cells.

SWELLING: When washing was completed (final recovery of cells, 85–90%), each group was recombined in 40 ml of GM into a separate tube and pelleted, accelerating to 1,500 rpm (530 *g*) at 1 min. The cell pellets (approximately 2.5 ml each) were each resuspended to 40 ml with ice-cold, 0.02 M Tris-HCl buffer, pH 7.4, and the cells were allowed to swell at 0°C for 5–15 min (27).

HOMOGENIZATION: The cells were then pelleted as above and the pellets were resuspended to 6 ml with 0.02 M Tris and transferred to two 8-ml Dounce homogenizers on ice. Each centrifuge tube was rinsed with an additional 2 ml of 0.02 M Tris, which was added to the appropriate homogenizer. Each homogenizer was then sealed with a clean piece of Parafilm and mounted in a chilled rubber adapter (no. 302, Dupont Instruments, Sorvall Operations, Newtown, Conn.) which had a 1.5-cm thick gauze cushion inserted in the bottom. The two homogenizers were then centrifuged for 3 min, accelerating to a plateau value of 2,070 rpm (1,000 *g*) by 1.5 min. The supernates were removed as completely as possible by aspiration, and the swollen, packed cells were disrupted on ice by one to three strokes with a separate tight-fitting Dounce pestle for each homogenizer (>95% cell breakage). Preparation of homogenates in this manner yielded vacuole suspensions which were concentrated enough ($>10^9$ vacuoles/ml) to permit adequate vacuole-vacuole contact in vitro; the homogenates were thus suitable for use without further experimental manipulation. It might be noted that the only noncellular substance present in the homogenates (besides the marker particles) is a small amount of Tris buffer.

IN VITRO INCUBATION: Aliquots (usually 50 μl) of each phagolysosome-rich homogenate were combined in 12 × 75-mm test tubes on ice; the tubes were vortexed for 5 s and incubated under various conditions. The fusion reaction was stopped by dilution (1:10 or more) with ice-cold 0.84% NaCl which was 10 mM in potassium phosphate buffer, 1 mM in $CaCl_2$, and 1 mM in $MgSO_4$, pH 6.8 (PBSM). Tubes were vortexed for 5 s and placed on ice.

DETECTION OF HYBRIDS: The mixtures were examined by light and electron microscopy as described further below.

Experiments with PS and PVT Particles

Early experiments, in which PS and PVT particles were used, followed the protocol given above, with these differences: the PS particles were added to the cultures at 1 mg/ml (29) and the PVT particles were used at 0.5 mg/ml. In successful experiments with these particles, the uptakes were about 100 PS particles/cell and about 30 PVT/cell. In searching for optimal conditions, the number of homogenization strokes was varied in different experiments (up to 20 strokes). The in vitro fusion reaction was stopped by dilution (1:11) with cold 30% (wt/vol) sucrose which was 0.02 M in Tris-HCl, pH 7.4, and the reaction mixtures were analyzed on linear sucrose or mannitol density gradients (density PS = 1.05; density PVT = 1.027). For each sample, 1 ml of the diluted reaction mixture was placed in the bottom of a 5-ml cellulose nitrate tube on ice. A linear gradient of 5–20% (wt/vol) sucrose or 6–18% (wt/vol) mannitol (both containing 0.02 M Tris, pH 7.4) was then layered over each sample with a polystaltic pump (Buchler Instruments, Inc., Fort Lee, N. J.) connected to a Buchler triple-outlet gradient mixer. Sets of six tubes were centrifuged at 4°C in a Beckman SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 42,000 rpm (2.1×10^5 *g*) for 15 min at speed in a Beckman model L-2 ultracentrifuge, which brought the buoyant PS and PVT vacuoles up to their equilibrium densities (cf. reference 29). Gradient fractions (1–1.5 ml) were collected with a J-shaped 23 G syringe needle. Fractions

were assayed by light microscope for PS, PVT, and associated PS + PVT particles by using a Petroff-Hausser bacteria counting chamber (Arthur H. Thomas Co., Philadelphia, Pa.) to determine the total particle concentration in each fraction, followed by oil immersion counts to determine the percent of each type present (PS, PVT, or PS + PVT). Determination of percents was based on two or three independent samplings of each fraction with at least 500 particles counted per sampling (1,000–2,000 total counts/fraction). Gradient fractions were examined by EM as described below.

Light Microscopy

Unfixed samples of in vitro reaction mixtures in PBSM were observed directly in slide and cover slip preparations and photographed at various magnifications (up to 800) on a Zeiss Universal microscope. In dye exclusion experiments, a 2% solution of eosin Y (Harleco, Philadelphia, Pa.) in PBSM was filtered through a 0.45-μm pore Millipore filter and mixed with vacuole suspensions at 0°C at various dilutions, usually 1:3 or 1:5. Color photomicrographs were made with high speed. Ektachrome B using a 550-nm short wave pass interference filter (Optics Technology, Inc., Palo Alto, Calif.) between the lamp diaphragm and the condenser, which optically converted the magenta eosin stain into a dark blue. A time-lapse sequence of vacuole membrane rupture and dye penetration into the markers was made manually with the same equipment.

Electron Microscopy

Portions of the in vitro reaction mixtures containing yeast- and GGRBC-labeled vacuoles which had been diluted with PBSM were diluted 1:1 with unbuffered 2% OsO_4 which contained 0.85% NaCl, 1 mM $CaCl_2$, and 1 mM $MgSO_4$. They were allowed to fix for 1–2 h or for extended periods (up to 72 h) at 0°C. By oil immersion phase microscopy and by EM, no significant differences were detected between samples of this type fixed for long or short periods at 0°C. After fixation, samples were washed twice with PBSM, dehydrated through a graded ethanol series, treated with propylene oxide, and embedded in Epon.

Gradient samples (1–1.5 ml) containing PS- and PVT-labeled vacuoles were diluted 1:1 with 3% glutaraldehyde which contained 0.2 M sucrose and 1 mM $CaCl_2$, and were buffered with either 0.05 M cacodylate, pH 7.2, or 0.05 M sodium phosphate, pH 6.9. Samples were fixed for 1–3 h or overnight at 0°C, after which they were diluted to 5 ml with 6% (wt/vol) mannitol and pelleted at 35,000 rpm (1.5×10^5 *g*) for 30–45 min in the Beckman SW50.1 rotor. The pellets were then processed for EM essentially according to Wetzel and Korn (29), with the addition of 0.25 M sucrose or mannitol to the solutions before use.

After embedded samples had polymerized at 60°C for 12–48 h, silver to gold thin sections were cut with a DuPont diamond knife (E. I. DuPont de Nemours and Co., Wilmington, Del.) on an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.). Sections were picked up on bare copper grids and double-stained with ethanolic uranyl acetate (8) and Sato's lead stain (10), usually for 10 min each. Grids were examined in a Hitachi HU-11B electron microscope at 75 kV. Ribbons of serial sections were picked up on 0.3% parlodion films supported on copper grids with 1 × 2-mm slots. They were stained with lead and examined at 75 kV.

Electron microscope counts of reaction mixtures containing yeast and GGRBC were made by cross sectioning pellet fragments from top to bottom and mounting the large sections on 85-μm slot parallel-bar grids (no. R-200, Ernest F. Fullam, Inc., Schenectady N. Y.) with the depth of the sample running approximately parallel to the grid bars. Counts were made by scanning "vertically," moving parallel with the grid bars; when a vertical area was finished, the field was shifted horizontally to a new grid bar, where the vertical counts were resumed. Thus, the sample was scanned in two dimensions, which substantially compensates for the nonrandom distribution of particles in the pellets (see reference 2). It was found that the vacuole membrane profiles were clearly contrasted against the yeast cell walls and were easily visible, whereas the membranes around the GGRBC were often very tight and considerably more difficult to see at low magnification. Therefore, all counts were made relative to the yeast population. (Yeast and GGRBC particles are present in approximately equal numbers in the reaction mixtures.) The yeast-profile population was classified into three types of profiles: (*a*) bare yeast, a yeast profile not surrounded by a membrane profile (or only partially surrounded); (*b*) yeast vacuoles, one or more yeast profiles completely surrounded by a membrane profile; (*c*) hybrid vacuoles, a yeast vacuole containing one or more GGRBC profiles. Typically, each pellet was sectioned in one or more places and 1,400–2,500 counts were made to determine the percent of each type of profile present.

RESULTS

In Vivo Fusion Experiments

Before attempting to develop an in vitro vacuole fusion system, in vivo experiments were carried out to extend the in vivo observations of Wetzel and Korn (29).

Phagocytosed particles (all types tested) were observed initially to be scattered throughout the cytoplasm of *Acanthamoeba* cells. They were seen by phase microscopy to combine gradually, presumably by the mechanism of vacuole fusion, into larger cytoplasmic groups, as reported by Wetzel and Korn for PS particles (29). This appeared to be an irreversible process. All of the phagocytosed

particles in an individual cell were frequently observed to have accumulated into a single large vacuole 3–4 h after particle uptake. To examine the mechanism of particle segregation in more detail, the cells were sequentially pulsed with 0.8-μm PS and 2-μm PVT particles, with samples for light and electron microscopy being taken at various times (see Methods). Light microscope examination of the cells just before the addition of the PVT particles showed that virtually all of the uningested PS particles had been removed by the washing procedure. At this time (45 min after the initial exposure to the PS pulse), the majority of the ingested PS particles (about 10/cell) were grouped in small cytoplasmic clusters of three to six particles per cluster. Electron microscope examination of cells fixed 15 min after the addition of the PVT particles (sample taken at 7 min) provided convincing evidence for the in vivo fusion of PS- and PVT-labeled vacuoles. A plausible sequence of fusion events is shown in Fig. 2. It should be emphasized that the sequence shown is an interpretative one, based on the static images of the fusion process recorded in different cells. Morphologically, it appears that after the vacuoles come into close apposition (Fig. 2 *a*), the fusion process is initiated by the formation of a small intervacuolar channel at a point of close contact (Fig. 2 *b*). This is apparently followed by a progressive widening of the newly formed junction, concomitant with a transition from a "hourglass" profile of the vacuole (Fig. 2 *c*) to a less constricted profile (Fig. 2 *d*). Similar results were obtained using GGRBC and yeast particles as markers (Fig. 3).

In Vitro Experiments (Qualitative)

Light micrographs of typical homogenates of GGRBC-containing and of yeast-containing cells are shown in Fig. 4. When aliquots of these homogenates were mixed and incubated in vitro fusion of the vacuoles occurred, as evidenced by the appearance of a new class of vacuoles containing both types of marker particles (hybrids) (Fig. 5). Controls are given below. To strengthen the interpretation of the phase-dense line surrounding the marker particles (Fig. 5) as indicating the presence of a membrane, and to test simultaneously the functional state of the vacuolar membrane, light microscope experiments were done with the dye eosin Y which is well known not to penetrate the plasma membrane of viable cells (1). As expected, live amebas, live yeast, and fresh red blood cells excluded the dye, whereas after treatments which are known to damage the integrity of cellular membranes (freezing and thawing, chemical fixation, autoclaving), each of these cell types freely admitted the dye. Thus, all GGRBC and autoclaved yeast particles from stock solutions readily stained with the dye, either at 0°C or at room temperature. However, the same particles, when surrounded by the phase-dense line, did not stain with the dye. The property of dye exclusion was sensitive to the observation conditions. Samples observed after being kept on ice in the presence of dye maintained the dye-exclusion ability (during initial observation), while samples from the same tube which had been placed on the microscope stage (at room temperature) and observed under oil immersion, often lost the ability to exclude the dye after a few minutes of observation, usually preceded by a sudden rupture of the phase-dense boundary (Fig. 6).

The membranous nature of the enclosing boundary was further confirmed by electron microscopy of the same samples: the only bounding structure seen was an approximately 90-Å membrane (Fig. 7). No change in the fine structure of the membranes of hybrid vacuoles was apparent by EM (Fig. 7 *c*), a finding consistent with the dye exclusion results. Three-dimensional closure of intact vacuolar membrane profiles was confirmed by serial sectioning completely through hybrid vacuoles (Fig. 8). In vitro formation of hybrid vacuoles was also obtained with PS- and PVT-labeled vacuoles (see below), as well as with PVT- and yeast-labeled vacuoles (not shown).

Mechanism of Hybrid Formation In Vitro

Several mechanisms other than vacuole membrane fusion can be hypothesized to give rise to hybrid vacuoles in these reaction mixtures (Fig. 9). Beside the fact that there are apparently no experimental precedents to support any of the last three mechanisms shown in Fig. 9, fusion can be experimentally distinguished from these alternative mechanisms since fusion requires the simultaneous presence of membrane around both marker particles, while the other mechanisms do not. For this purpose, controls were routinely included in which yeast particles were added directly to aliquots of GGRBC-homogenates, and GGRBC particles were added to yeast-homogenates; these control tubes were incubated under the same conditions as the in vitro reaction mixtures of the

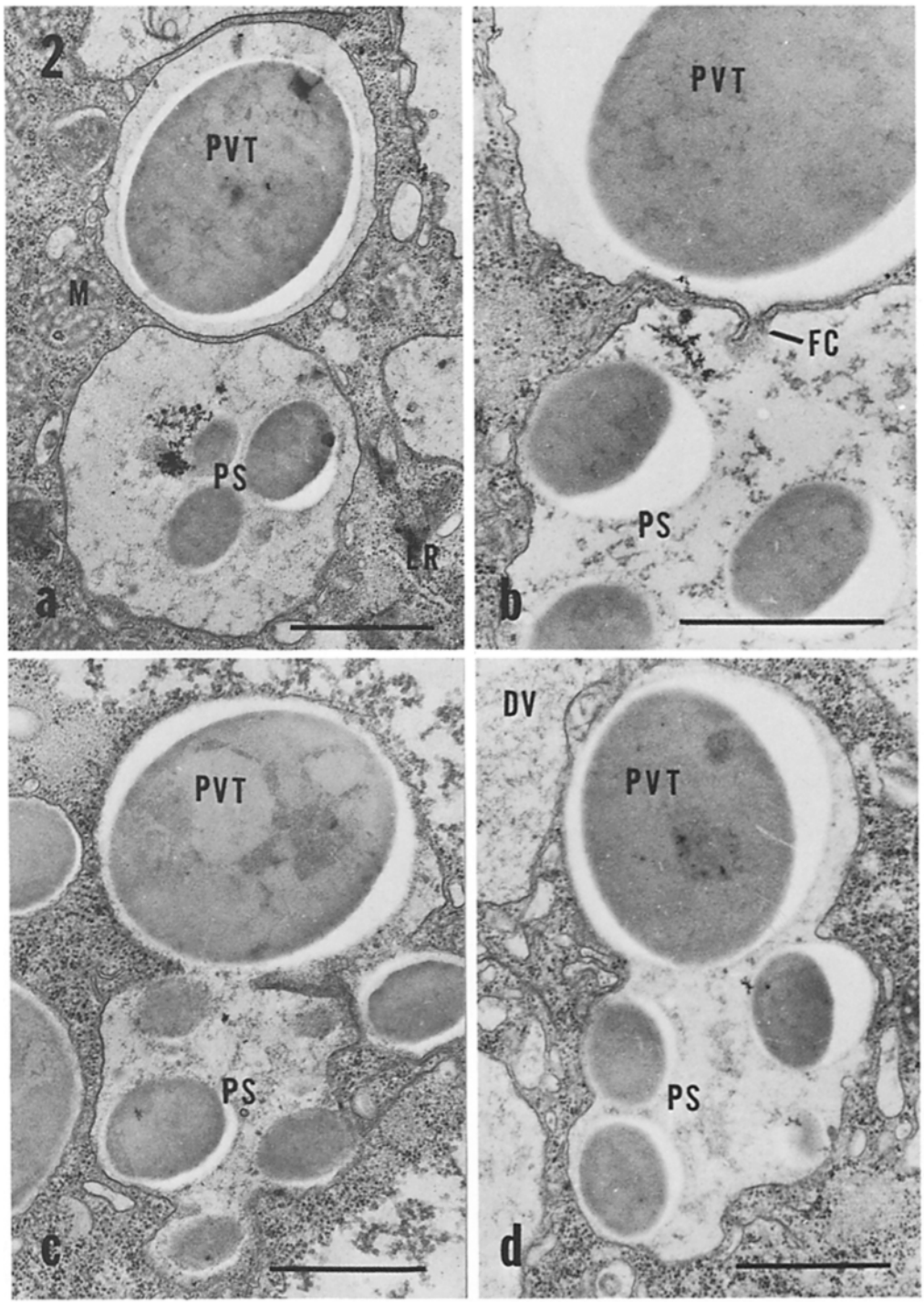


FIGURE 2 In vivo fusion of vacuoles labeled with PS particles (PS) and PVT particles (PVT). The cells were pulsed with PS particles, washed, then pulsed with PVT particles, and fixed. Several apparent stages of the fusion process can be seen. The elliptical cross sections of the spherical particles are due to particle compression during sectioning, which also causes some separation of the particle profiles from the surrounding Epon (white areas). The amorphous material within these vacuoles was derived from the culture medium, as evidenced by its absence in subsequent experiments in which "particle-free" medium was employed (see Methods). See text for further description. *FC*, fusion channel; *M*, mitochondrion; *DV*, digestive vacuole; *ER*, endoplasmic reticulum. Bars = 1 μm. (*a*) × 21,000; (*b*) × 30,000; (*c*) × 23,000; (*d*) × 22,000.

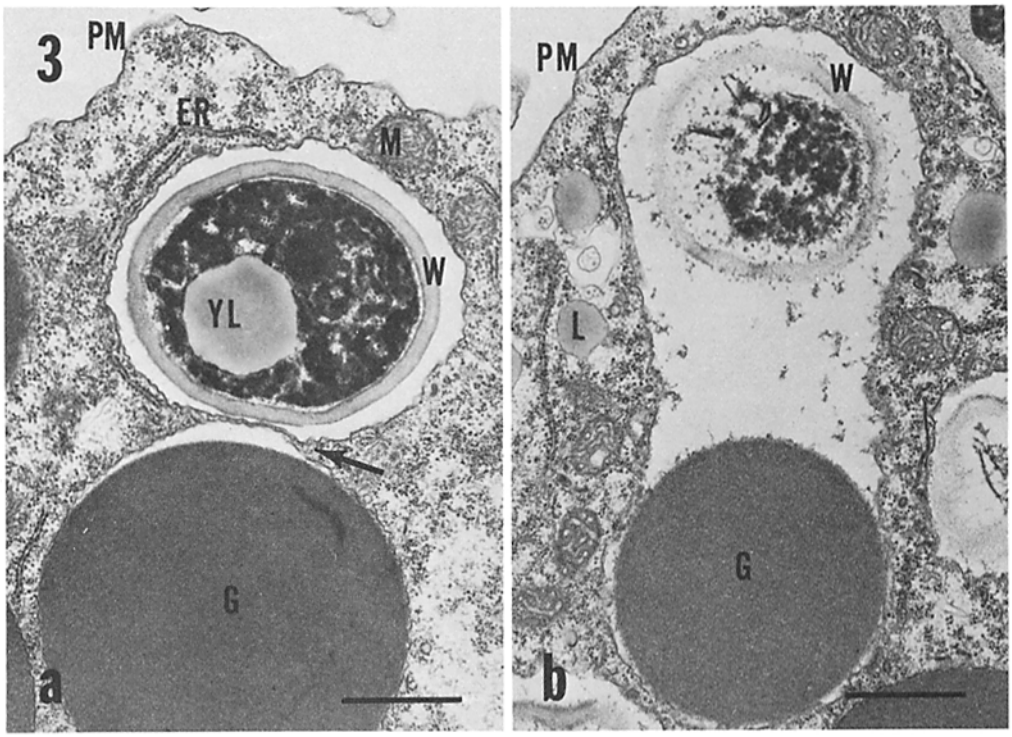


FIGURE 3 In vivo fusion of vacuoles labeled (as in Fig. 2) with GGRBC particles (*G*) and autoclaved yeast particles. The membranes of a yeast and a GGRBC vacuole are seen in close apposition in Fig. 3 *a* (arrow). Note the absence of amorphous material in these vacuoles. In Fig. 3 *b*, continuity is seen to be established between a yeast and a GGRBC vacuole. The flocculent material within the hybrid vacuole appears to result from the partial digestion of the yeast matrix. *W*, yeast cell wall; *YL*, yeast lipid droplet; *PM*, ameba plasma membrane; *M*, mitochondrion; *ER*, endoplasmic reticulum; *L*, lipid droplets. Bars = 1 μm. × 16,000.

two homogenates. The same types of controls were also done with PS and PVT particles in experiments in which these particles were used as markers. The number of particles added to the control tubes was such that the concentration of the bare particles was approximately equal to or in excess of the same particles in the two-homogenate reaction mixtures (approximately 2×10^9/ml of each type). In all cases, these controls were completely negative: checked by electron microscopy, the added particles did not acquire membranes during the in vitro incubation, nor did they penetrate the membranes of the pre-existing vacuoles, i.e., no hybrids were formed. With the GGRBC and yeast particles, these controls were also checked by light microscopy: after the incubation, the addition of eosin Y stained 100% of the added particles. These control experiments indicate that the presence of membrane around both markers is required for the formation of hybrid vacuoles in vitro. This finding directly points to membrane fusion as the mechanism of hybrid vacuole formation. Furthermore, convincing evidence that hybrids are formed by membrane fusion was found in the EM appearances of many of the hybrid vacuoles in which the particles were tightly surrounded by their membranes (Figs. 10, 11 *a*, *b*).

A final consideration concerning the small percentage of whole cells which survive homogenization should be mentioned. Although unlikely, the hypothesis might be entertained that the whole cells surviving homogenization which are present in the reaction mixtures could be responsible for the formation of hybrids: hypothetically, a GGRBC-labeled cell could phagocytose a yeast particle during the in vitro incubation with subsequent yeast and GGRBC vacuole fusion occurring in vivo, followed by a spontaneous release of the hybrid vacuole from the cell. This hypothesis is

ruled out by the fact that no hybrids were formed in the control experiments described above. Furthermore, remaining cells were carefully examined by phase and electron microscopy after the incubation. They were found, in all cases, to contain only one type of particle, which indicated that they were incapable of phagocytosing "opposite" particle types under the experimental conditions. Therefore, it is concluded that the mechanism of hybrid vacuole formation in vitro is vacuole membrane fusion in vitro.

Morphology of In Vitro Vacuole Fusion

Morphologically, the fusion mechanism in vitro appears to be the same as that observed in vivo, with fusion being initiated at vacuole contact points by the formation of a small channel (Figs. 10, 11 *b*, *c* [cf. Fig. 2 *b*]). Multiple-site fusion, i.e. simultaneous fusion at more than one site of a vacuolar membrane, was observed to occur in vivo (see also micrographs in reference 3), but at a considerably lower frequency than single-site fusion. In the in vitro reaction, however, in addition to single-site fusion, multiple-site fusion was also commonly observed (Fig. 11). In Fig. 11 *a*, fusion is seen at two main sites of the GGRBC vacuole membrane, with the membrane between the fusion sites forming an angled, "flattened vesicle" in cross-sectional appearance. In some cases (Fig. 11 *b*), a lacelike network of fine connections between vacuoles was seen, indicating extensive multiple-site fusion. More commonly, where the mem-

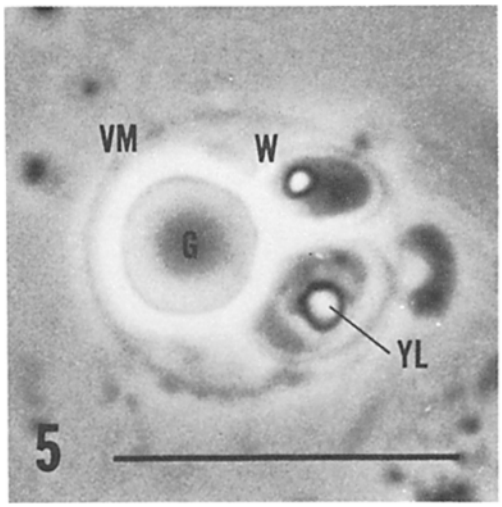


FIGURE 5 A hybrid vacuole formed by in vitro incubation of the two homogenates. A refractile GGRBC particle (*G*) and two yeast particles are seen to be bounded by a continuous phase-dense boundary line (vacuole membrane [*VM*]). The unfixed, unstained vacuole was photographed in suspension with oil-immersion phase optics. *W*, yeast cell wall; *YL*, yeast lipid droplet. Bar = 10 μm. × 4,600.

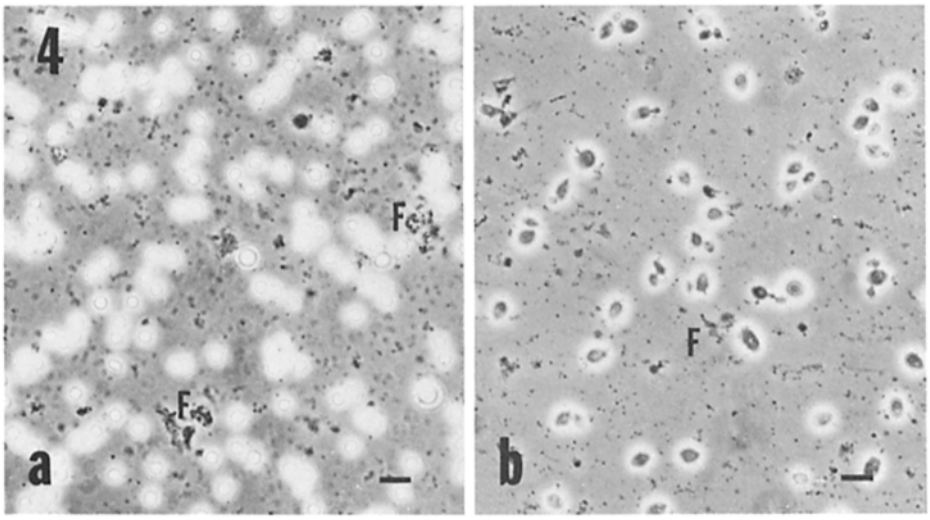
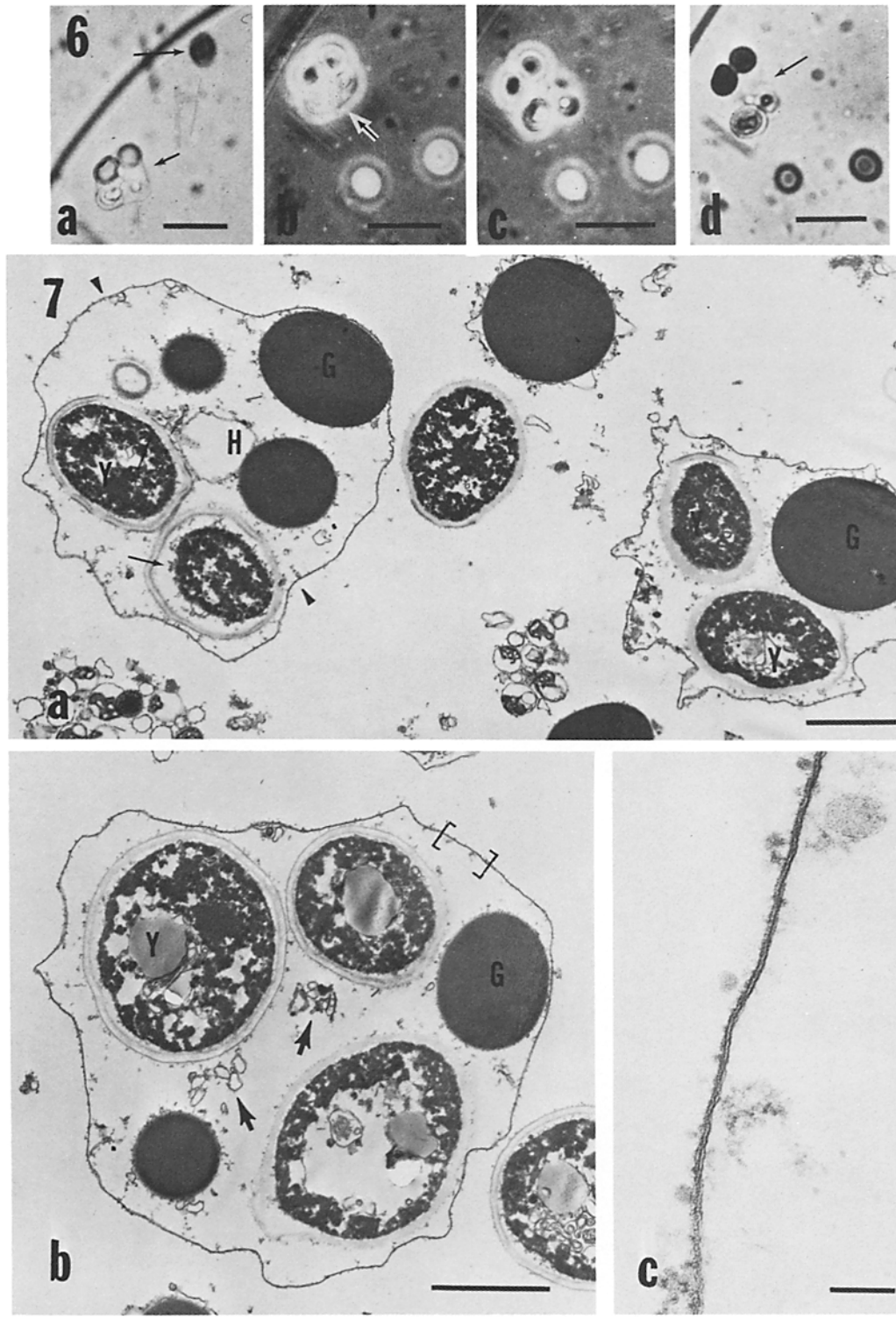


FIGURE 4 Light micrographs of PBSM-diluted homogenates of cells whose vacuoles were labeled with highly refractile GGRBC particles (Fig. 4 *a*) or with yeast particles (*b*). The membranes around the particles are not visible at this magnification. Many of the small dark granules are mitochondria. Slide and cover slip preparations. *F*, cell fragments. Bars = 10 μm. Phase contrast. × 440.


6
a
b
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G
H
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Y
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c

branes did not surround the particles quite so tightly, multiple-site fusion was evidenced by the presence of apparent "internal vesicles" in the region of fusion (Fig. 11 *c*, *d*).

A finding of special interest is that, despite extensive scrutiny of the in vitro homogenate mixtures by electron microscopy, fusion has been seen to occur only between phagolysosomes. Fusion of mitochondria or nuclei with phagolysosomes (or with themselves or each other) was not detected. Similarly, despite the recent origin of a substantial portion of the phagolysosomal membrane from the surface membrane, no fusion of particle-labeled vacuoles with the plasma membrane of unbroken cells or cell fragments was observed. While it was not possible to determine reliably the in vitro fusion behavior of other subcellular membranous components in these experiments, the appearance of these components in samples kept on ice and in samples incubated for fusion was not noticeably different.

In Vitro Experiments (Quantitative)

In early experiments with PS and PVT particles, the in vitro reaction mixtures were analyzed on density gradients (see Methods). This technique proved capable of detecting very low (0.05%) amounts of fusion by virtue of the hybrid densities of the hybrid vacuoles. Gradients were centrifuged at 42,000 rpm for 15 min; longer centrifugation times (30, 60, or 90 min) caused no noticeable change in the synthetic particle distribution. A typical banding pattern is shown in Fig. 12. As expected, the I region (see Fig. 12) was the most enriched in PS + PVT hybrids, but the predominant vacuole type in this region was unassociated PVT vacuoles which equilibrated at lower density than the main PVT population. Rebanding of the vacuoles from this fraction in a second density gradient did not alter the particle distribution.

The quantitative effects of in vitro incubation time and temperature on the appearance of "associated" PS + PVT markers in the I region were determined by light microscope counts, as described in Methods. The results from a typical experiment are shown in Fig. 13. A rapid rise in the level of associated PS + PVT was observed during the first 10 min of in vitro incubation at 30°C. The appearance of the PS + PVT was strongly temperature dependent and unaffected by the presence of 20 μM 2,4-dinitrophenol (triangle symbol in Fig. 13). The phenomenon of the decrease in the PS + PVT level after 10 min, which could be interpreted in several different ways, was clarified in later experiments (see below). Although the level of PS + PVT hybrids varied somewhat from experiment

FIGURE 6 Time-lapse sequence of membrane rupture of a hybrid vacuole in the presence of eosin Y. In Fig. 6 *a*, a hybrid vacuole (formed in vitro) with two GGRBC and two yeast particles is seen to exclude the dye. The membrane appears as a faint line surrounding the particles (short arrows). Note the nearby (moving) particle which is stained (long arrow). The heavy diagonal line is an artifact of the photographic system. When the optics are switched from bright field (Fig. 6 *a*) to phase contrast (with removal of the 550-nm filter), the membrane of the same vacuole appears as a phase-dense boundary (Fig. 6 *b*, arrow). Presumably due to the absence of the light- and heat-absorbing filter (see reference 1), the phase-dense boundary is subsequently seen to disappear suddenly (rupture), with the release of physical constraint on the particles (Fig. 6 *c*). When the optics are returned to bright field (with the filter reinserted), the boundary is seen to be absent and the particles are now intensely stained (Fig. 6 *d*). Some apparent remnants of the membrane are visible at the arrow. Approximately 90 s between pictures (the actual rupturing event occurs in a fraction of a second). Black and white prints of color photographs. Bars = 10 μm. (*a*) × 970; (*b*); (*c*); (*d*) × 1,160.

FIGURE 7 Electron micrographs of hybrid vacuoles formed in vitro. Groups of yeast (*Y*) and GGRBC (*G*) particles are seen to be bounded by continuous membrane profiles. In Fig. 7 *a*, the larger hybrid appears to have formed by fusion at two "peripheral" sites (along the line indicated by the arrowheads), creating a central vesicle, invagination, or hole (*H*), which probably corresponds to the small central intravacuolar ring of membrane that was often observed by light microscopy (e.g., Fig. 6 *a*). The matrix of one of the yeast particles appears to be partially digested (arrow) (cf. Fig. 3 b). In Fig. 7 *b*, small intravacuolar membrane profiles, which were frequently observed in the hybrid vacuoles, are seen (arrows). The bracketed portion of the vacuolar membrane is shown at higher magnification in Fig. 7 *c*. Typical unit membrane structure is evident. (*a*) × 6,900; (*b*) × 9,100; bars = 2 μm. (*c*) × 106,000; bar = 0.1 μm.

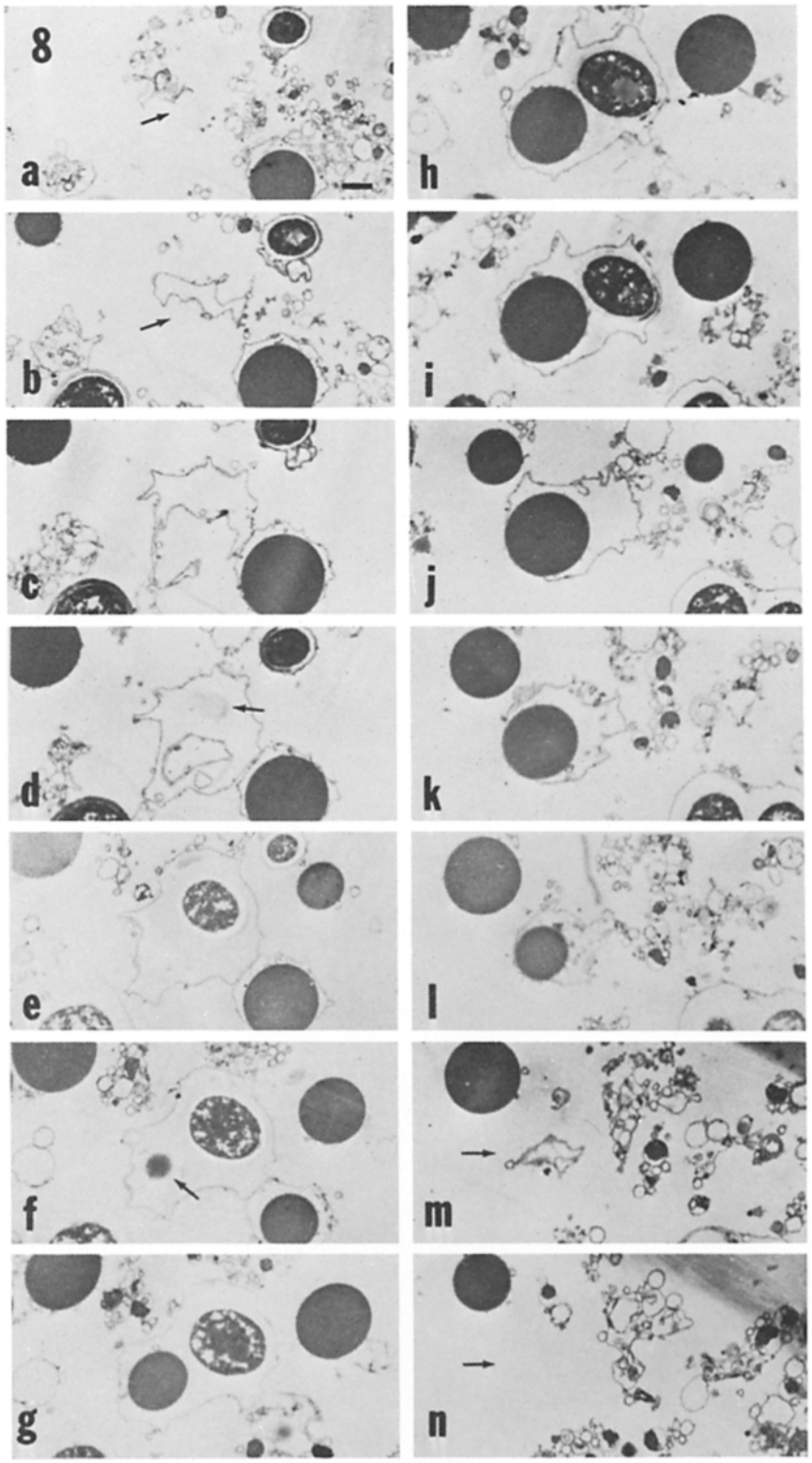

FIGURE 8 Serial sections through a hybrid vacuole formed in vitro. One edge of the vacuolar membrane lies between 8 *a* and 8 *b* (arrows). Sectioning continues through an invagination (8 *c*, 8 *d*), a yeast particle (8 *d* [arrow] and 8 *e–i*), and a GGRBC particle (8 *f* [arrow] and 8 *g–l*). The other edge of the vacuole lies between 8 *m* and 8 *n* (arrows). Approximately every fourth section is shown. Bar = 1 μm. × 5,600.

to experiment (see Discussion), and while in these early experiments the overall hybrid levels were relatively low (about 1% at 10 min), the pattern shown in Fig. 13 was consistently observed. It

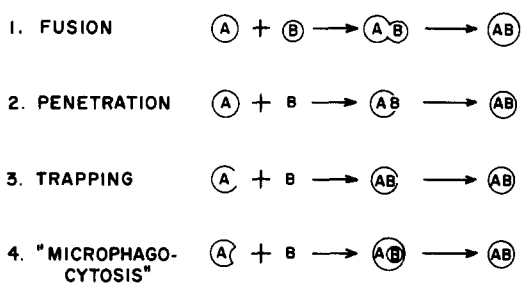

FIGURE 9 Hypothetical mechanisms of hybrid vacuole formation. Hybrid vacuole formation could result from several conceivable processes. These include (*a*) membrane fusion, (*b*) direct penetration of a particle into a vacuole, (*c*) closure and resealing of a ruptured vacuole (or membrane fragment) to include both particle types, and (*d*) entry of a particle into a vacuole by invagination and pinching off of the vacuole membrane, with subsequent dissolution of the internalized membrane.

should be pointed out that under the conditions of the light microscope assay used, it was not possible to discriminate fused PS + PVT vacuoles from marker combinations possibly resulting from simple adhesion. However, EM examination of these fractions showed fused PS + PVT vacuoles (e.g., Fig. 11 *c*) to occur in relative frequencies (judged qualitatively) which corresponded with the pattern determined by light microscopy. Furthermore, as described in the following paragraph, this pattern was subsequently confirmed by EM counts of whole reaction mixtures of GGRBC and yeast vacuoles with substantially higher fusion levels.

EM counts of whole reaction mixtures were made relative to the yeast population as described in Methods. The term "total yeast population" is used here to mean the sum of the profile counts of bare yeast, yeast vacuoles, and hybrid vacuoles for a given sample. The results of an in vitro fusion experiment analyzed in this way are given in Fig. 14. Fig 14 *a* shows the percent of yeast-GGRBC hybrid vacuole profiles relative to the total yeast population (TYp) as a function of in vitro incuba-

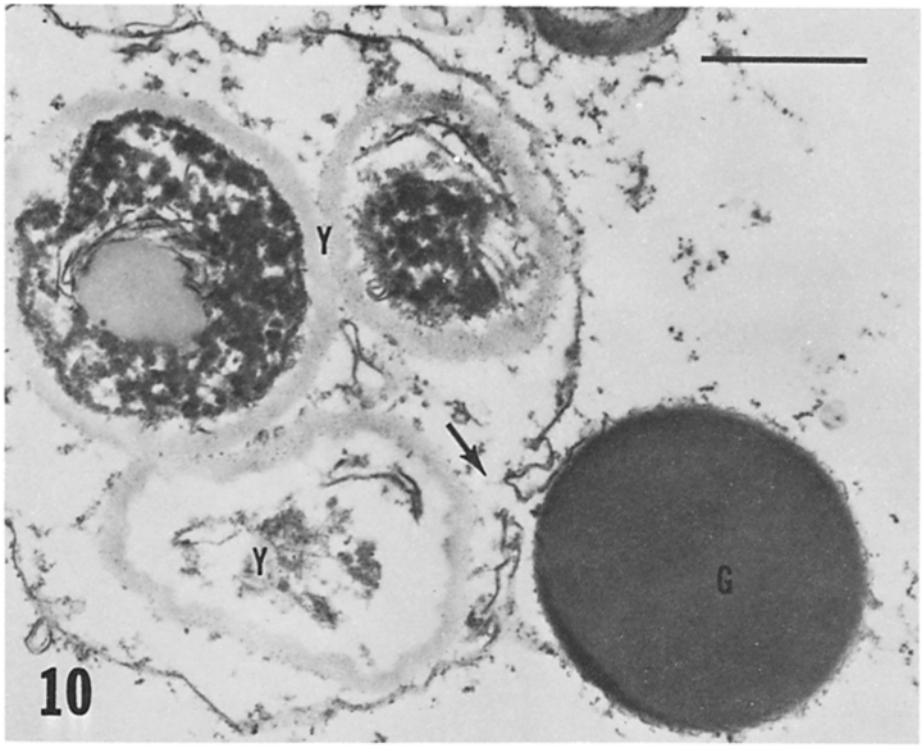

FIGURE 10 In vitro formation of an apparent fusion channel. A vacuole with three yeast profiles (*Y*) (in varying stages of digestion) is seen to be connected by a small fusion channel (arrow) to a GGRBC vacuole (*G*) with a tight-fitting membrane. The flocculent material outside of the vacuole probably originates from the digestion of other yeast particles in the sample. Bar = 1 μm. × 22,000.

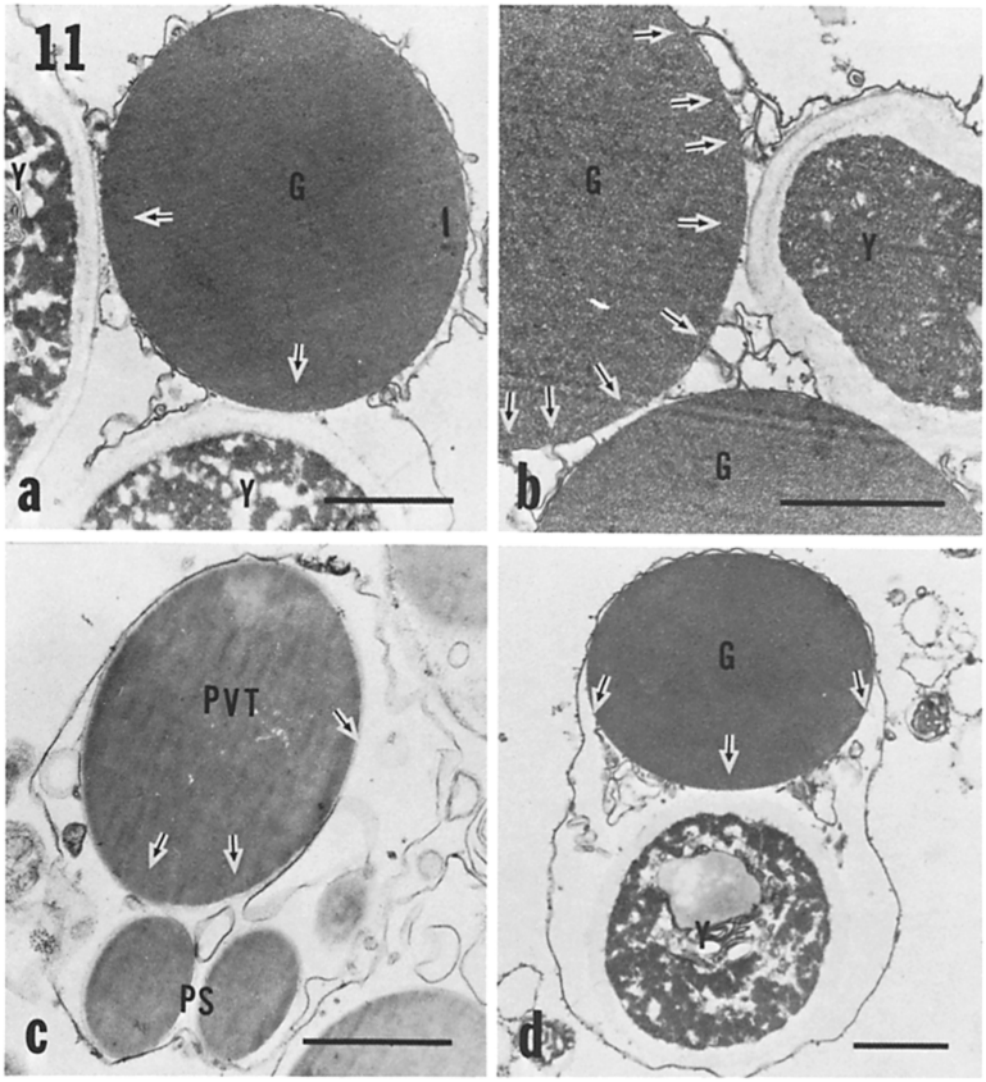

FIGURE 11 Examples of different vacuole morphologies arising from multiple point fusion. Arrows indicate sites of fusion. See text for discussion. *G*, GGRBC particle; *Y*, yeast particle; *PS*, PS particle; *PVT*, PVT particle. Bars = 1 μm. (*a*) × 17,000; (*b*) × 22,000; (*c*) × 20,000 (*d*) × 13,000.

tion time and temperature. The resulting pattern is quite similar to that determined by light microscopy with the PS-PVT gradient system (Fig. 13), confirming that the reaction is time and temperature dependent and that it peaks at about 10 min. Additionally, a slower phase of hybrid vacuole breakdown is seen after 20 min of incubation. The same type of lysis pattern was also found for the yeast vacuoles (Yv) in the same samples (Fig. 14 *b*). This indicates that the observed breakdown of the hybrid vacuoles (Fig. 14 *a*) is not due to an inherent instability of the hybrids, but reflects a lytic process which affects the entire vacuole population (hybrid and nonhybrid) in the same way. In Fig. 14 *b*, the lytic phenomenon is also seen to be temperature dependent. It should be noted that the precise agreement between the 0°C points in this figure is somewhat fortuitous, since the precision of this technique was only about ±10–15% in this range of values (e.g., 40% ± 6%). Although a small decrease in the percent of yeast vacuoles is expected as a consequence of the

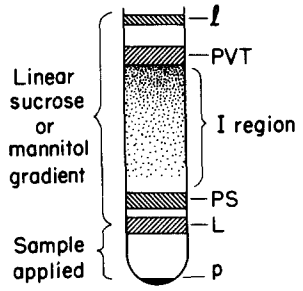

FIGURE 12 Typical banding pattern of PS- and PVT-labeled vacuoles in linear density gradients. *l*, lipid droplets; *PVT*, main PVT vacuole band; *I* region, hybrid density region with "heavy" PVT vacuoles and PS + PVT hybrids; *PS*, main PS vacuole band; *L*, interface of gradient and applied sample; *p*, pellet.

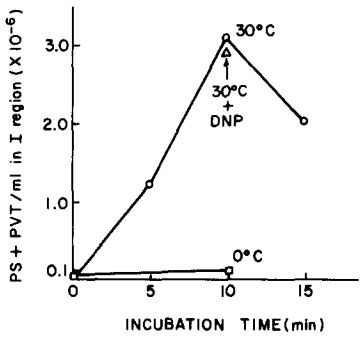

FIGURE 13 Typical pattern obtained from light microscope counts of PS + PVT hybrids in the I region as a function of incubation conditions. The 2,4-dinitrophenol (DNP) concentration in this experiment was 20 μM.

formation of hybrid vacuoles (here about 5% at 10 min), this was not detected in this experiment (Fig. 14 *b*). This is apparently due to the fact that this relatively small change (5%/35%) is within the range of experimental error of this technique (above). Support for this interpretation is given by a more recent experiment in which samples with substantially higher hybrid levels were analysed by means of an improved EM sampling technique.[3] The EM results showed 55% yeast vacuoles at zero time with 0.07% hybrids (1 hybrid/1,444 counts). Duplicate reaction mixtures incubated at 30°C for 10 min gave 47% ± 2% yeast vacuoles and 9.6% ± 0.2% hybrids (mean ± average deviation, approximately 1,000 counts per sample).

[3] P. J. Oates and O. Touster, manuscript in preparation.

These values add up to give a total vacuole count after the 10-min incubation of 56.6% ± 2%, which is unchanged from the initial value. Of the total vacuole population (yeast + hybrid vacuoles), 17% were hybrids. Thus, a decline in the percent of yeast vacuoles was seen which was equal to the percent hybrids formed, with the total vacuole population remaining constant (within experimental error) during the first 10 min of incubation. These results, as well as the results of Fig. 14 *a* and *b*, indicate that vacuole breakdown is not occurring during the time in which fusion is taking place. From the data of Fig. 14 *a* and *b* the percent of hybrid vacuoles relative to the existing total vacuole population (TVp) can be obtained (Fig. 14 *c*). Although additional points in the transitional region are desirable, it appears that the fusion reaction progresses up to the point where vacuole rupture begins (about 10 min), at which time it abruptly ceases. The plateauing of the reaction after 10 min indicates that the yeast vacuoles and the hybrid vacuoles are rupturing (with no fusion occurring) at very similar rates (Fig. 14 *a*, *b*).

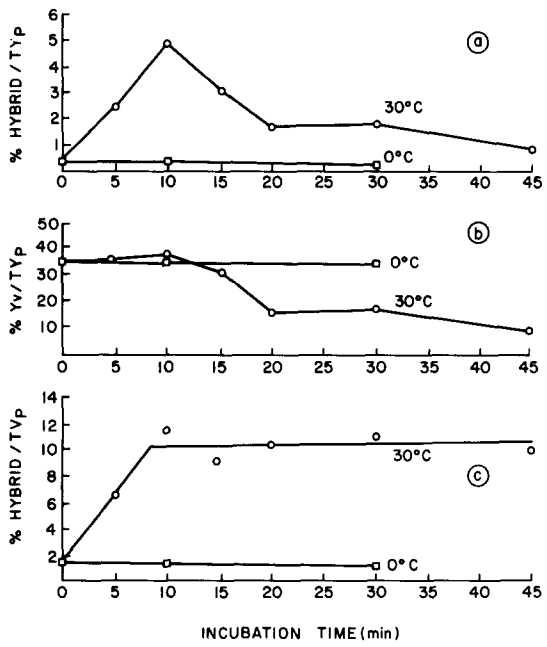

FIGURE 14 Electron microscope counts of hybrid vacuole formation (yeast + GGRBC markers) as a function of in vitro incubation time and temperature. *TYp*, total yeast population; *Yv*, yeast vacuoles; *TVp*, total vacuole population (Yv + hybrids).

DISCUSSION

Lysosomal Nature of the Vacuoles

The union of endocytotic vacuoles with lysosomes has been well documented in many cell types (see reference 6 for a review). Although the lysosomal system of *Acanthamoeba* has not yet been studied in detail, its essential features appear to be typical of those found in other phagocytic cells. In ingesting particulate matter greater than 1 μm in diameter, the ameba closely surrounds each particle with its plasma membrane (12, 28); the membrane-enclosed particle then buds off into the cytoplasm, giving rise to a newly formed phagosome (9, 29), which is observed to fuse subsequently with other members of the vacuolar system and to acquire a variety of hydrolytic enzymes (29). In sucrose gradients of *Acanthamoeba* extracts, Müller (17) has shown the presence of six sedimentable acid hydrolases, each with an almost identical distribution pattern which is distinct from the mitochondrial and peroxisomal patterns. These data strongly indicate the presence of lysosomes in *Acanthamoeba*. The data of Wetzel and Korn (29), who used an elegant isolation procedure for PS-containing phagososomes from *Acanthamoeba*, indicate that these newly formed phagosomes rapidly acquire lysosomal enzymes. After a 30-min incubation with PS particles, the highly purified phagocytic vacuole fraction was found to contain 20–25% of the total acid phosphatase and about 15% of the acid β-glucosidase, α-glucosidase, and β-galactosidase (29). Acid phosphatase and α-glucosidase are two of the six lysosomal enzymes identified by Müller (above). These results indicate a relatively rapid entry of phagocytosed particles into *Acanthamoeba's* lysosomal system, with many of the phagosomes becoming phagolysosomes soon after ingestion. This conclusion is supported by numerous EM observations and various enzyme determinations made in the course of this work, as well as by EM histochemical results on phagocytosing cells and isolated vacuoles (P. J. Oates and O. Touster, unpublished observations). The observed disintegration of the internal matrix of many of the intravacuolar yeast particles is also consistent with the presence of intravacuolar digestive enzymes (Figs. 3 *b*, 10). (The cell walls of the autoclaved yeast, which appear by EM to be highly porous, are resistant to digestion by *Acanthamoeba* [18].) Since the washing procedure at 30°C in GM takes about 1.5–2 h after the initial ingestion of the particles, and since it was observed that vacuole fusion continues to occur in vivo during this time, it is quite likely that the majority of the particle-containing vacuoles also contain lysosomal enzymes by the time that the homogenates are prepared for the in vitro experiments.

Development of the In Vitro System

The foundation for attempting this study was largely laid by the work of Weisman and Korn (28) who observed that PS and PVT beads were ingested by *Acanthamoeba*, and by the subsequent work of Wetzel and Korn (29) who reported observing the in vivo fusion of PS-labeled vacuoles and showed that PS vacuoles could easily be isolated on sucrose step gradients. Since PS and PVT particles differ substantially in their densities, sucrose gradient analysis of in vitro mixtures of *Acanthamoeba* homogenates labeled with these two particles seemed to be a promising system with which to attempt to demonstrate in vitro fusion of the vacuoles. Although the PS-PVT gradient system served its initial purpose in providing a sensitive detection method for in vitro fusion, a number of problems associated with the use of these particles led to a search for alternative markers.[4]

As pointed out by Neff (18), the amebas avidly ingest autoclaved yeast cells. However, the relatively fast rate of sedimentation of the yeast makes it very difficult to separate the uningested yeast cells from the ameba cells by differential centrifugation. It is important to do this because in the in vitro mixture of the homogenates the physical presence of particles without membranes decreases the frequency of contact between particles with membranes. With the autoclaved yeast, this cell/particle separation problem was brought under control by the development of a procedure for preselecting a suitably sedimenting "light" fraction of the yeast population. Attention was then turned to finding a second marker particle. Various types of particles were tested but most were unsatisfactory for one reason or another. After noting literature reports that goat red blood cells were unusually small (23), experiments with these particles were undertaken. Glutaraldehyde fixation was employed to stabilize them for experimental handling and presumably to enhance their uptake by *Acanthamoeba* (24). After the fixation procedure

[4] One major problem was that the commercial production of the PVT particles was terminated while this work was in progress.

was developed, the GGRBC proved to be almost ideal.

Quantitative Aspects of the In Vitro System

Since fusion relies on vacuole contact, and vacuole contact in vitro depends directly on the concentration of vacuoles, it seems evident that the amount of hybrids formed in vitro will depend (up to a point) on the initial vacuole concentration in the reaction mixtures. Under the conditions of these experiments, the initial vacuole concentration is primarily a function of an effective but gentle homogenization procedure. Upon close examination, it appears that the swelling procedure needs to be carefully controlled, as a significant proportion of the cells can begin to lyse spontaneously in the hypotonic solution, followed presumably by lysis of their liberated vacuoles. Thus, in more recent experiments, shorter swelling times (5 min or less, see Methods) have been used with improved results. Comparison of the more recent data (given in Results) with those of Fig. 14 shows that, associated with an increase of approximately 60% in the percent of vacuoles initially present in the reaction mixtures (55% vs. 35%), a similar increase (70%) in hybrid vacuole formation was found (17% vs. 10%). This indicates that under the conditions of the experiment shown in Fig. 14, vacuole contact is probably a limiting factor in the in vitro fusion reaction.

The apparent constancy of the percent total vacuoles which was observed during the fusion reaction warrants comment. Taken at face value, this constancy of the percent total vacuoles could be interpreted to imply that fusion is occurring only between yeast vacuoles (Yv) and GGRBC vacuoles (Gv), and not between Yv and Yv, since Yv-Yv fusion should decrease the percent of yeast vacuoles and therefore the percent total vacuoles. However, the fact that fusion has also been observed between PS- and PVT-labeled vacuoles, as well as between other marker combinations such as yeast-PVT, indicates that fusion per se does not depend on the type of marker particles within the vacuoles. Furthermore, in Yv homogenates incubated alone for 10 min at 30°C, EM observations indicated that Yv-Yv fusion does take place in vitro. Consideration of the experimental conditions indicates that the extent of Yv-Yv fusion (as well as Gv-Gv fusion) should amount to roughly half of the hybrid level. Thus, it is quite likely that Yv-Yv fusion (and Gv-Gv fusion) does occur in the reaction mixtures, but that the quantitative effect of Yv-Yv fusion on the total vacuole level (TVp) is small enough to be within experimental error. If some source of bias were found to be operating against Yv-Yv fusion, it would probably be due actually to having a higher concentration of Gv than Yv present in the reaction mixtures, which would create such an apparent bias by lowering the relative Yv-Yv contact frequency. Alternatively, the possible simultaneous operation of other factors, such as vacuole fission (cf. reference 6), which could offset the expected small decrease in total vacuoles due to Yv-Yv fusion, would have to be examined.

It is evident from the above discussion that the hybrid levels may be expected to vary somewhat from experiment to experiment, depending primarily on the initial vacuole concentration in the homogenates. On the other hand, in a given experiment, the hybrid levels are found to be reproducible, which allows quantitative study of the reaction. Furthermore, since vacuole contacts are normalized in vitro, the reaction can be studied independently of vacuole contact requirements.

Regarding the overall quantitation of in vitro fusion, an additional point is that an undetermined percentage of hybrids (probably less than 20%) escape detection by EM due to the sectioning procedure employed. This consideration, coupled with the more important factor of the insensitivity of the assay method to Yv-Yv and Gv-Gv fusion, makes it likely that the number of hybrids detected reflects only about 50% of the "absolute" amount of vacuole fusion that is occurring. Thus a hybrid level of 15–20% probably reflects an overall vacuole fusion level of 30–40%.

Characteristics of In Vitro Fusion

Regarding the relationship between vacuole fusion in vivo and that observed here in vitro, several points may be noted: (*a*) in vitro vacuole fusion occurs here in the absence of any noncellular components (except for traces of Tris buffer); (*b*) it appears to occur by a morphological mechanism similar, if not identical to that observed in vivo; (*c*) as evidenced by the exclusion of dye by fused vacuoles and by the undamaged EM appearance of the membranes of fused vacuoles, in vitro fusion apparently occurs (see below) without significant membrane damage, as it presumably does in vivo (6); and (*d*) the specificity of vacuole fusion in vivo (5, 6) is apparently maintained in vitro. Taken together, these observations suggest that the in vitro reaction, at least in its initial stages, may resemble the in vivo reaction quite closely.

In contrast to the relatively well-controlled vacuole contact process in vivo (Figs. 2 *a* and 3 *a*), the increased freedom of vacuole-vacuole contacts afforded by the in vitro state would be expected (in hindsight) to lead to the higher frequency of multiple-site fusion observed in vitro. The occurrence of multiple-site fusion indicates that the membranes are capable of fusing at any given point where effective contact is made. A situation such as seen in Fig. 11 *a* presumably results from the simple contact geometry of a sphere contacting two other "spheres." Fig. 11 *b* indicates that the smoothness (or lack of it) of the contacting membrane surfaces also plays a role in the morphological outcome of the fusion process. Single-point contact at several sites is inferred to have occurred in Fig. 11 *c*, while Fig. 11 *d* is interpreted to show multiple-point contact at two sites. As clearly suggested in Fig. 11 *a–c*, most of the "internal vesicles" at fusion sites are most likely to be cross sections of the extravacuolar space bounded by the membrane in between fusion sites. However, this apparently does not apply to all membrane profiles within the vacuoles; many vacuoles also appear to contain numerous small vesicles and other membranous structures which are of undetermined origin. While it is topologically possible to generate true internal vesicles as a result of the fusion process, at present it does not seem possible to determine reliably the extent to which this might occur.

The observed temperature dependence of the fusion reaction is likely to be due to one or more of the following factors: (*a*) a requirement for enzyme action; (*b*) a requirement for a temperature-dependent phase state of the membrane components (cf. reference 22); or (*c*) an effect on the frequency of vacuole-vacuole contact. Further work to distinguish among these possibilities is presently being undertaken. At this time, it is not known whether any specific homogenate components are necessary for the fusion reaction to occur.

The cause(s) of vacuole breakdown and the reason it begins at 10 min and shows the pattern that it does are unknown at this time. More data are needed before deciding whether the breakdown curves reflect a single population decaying exponentially or whether there is a resistant subpopulation of vacuoles. The temperature dependence of vacuole rupture (Fig. 14 *b*) suggests that it is probably linked to an enzymatic process. Thus, one obvious possibility as to its cause is the direct action of hydrolytic enzymes (cf. reference 16) or their products (e.g., lysolecithin [14]) on the membranes. However, other possible factors, such as changes in osmolarity or pH, chelation of metal ions, or depletion of stabilizing factors must also be considered.

The observations reported here that membrane rupture was not detected while fusion was taking place, and that fusion appeared to cease with the onset of vacuole rupture, clearly suggest that membrane fusion precedes membrane rupture in this system. However, these observations do not bear directly on the question of membrane "lysis," where lysis is understood to mean the release of soluble cellular or vacuolar contents due to a transiet increase in membrane permeability (as occurs, for example, in hypotonic hemolysis [26]). Similarly, the observation here that the dye exclusion property of the vacuolar membranes is unimpaired after fusion has occurred does not rule out the possibility of a transient increase in membrane permeability while fusion is taking place. Thus, while the basic relationship in this system between vacuole fusion and vacuole rupture seems clear, a determination of the relationship between vacuole fusion and vacuole permeability will require further study.

The observed specificity of the fusion reaction in vitro suggests that the fusion specificity observed in vivo (6) is not achieved by a mechanism which prevents heterologous organelles from making close contact, but rather that the fusion specificity resides in the membranes of the different organelles (5–7). What makes the membranes of the lysosomal system different from other subcellular membranes in this regard is not known at the present time. However, this would appear to be an attractive system for future investigation, since *Acanthamoeba's* phagolysosomal membrane is chemically and enzymatically very similar to the plasma membrane (27), which appears to have relatively few components (13). In addition, a considerable amount of chemical and enzymatic data on these membranes has already been obtained by E. D. Korn and his group (4, 11, 13, 27).

Finally, the experimental approach described here would appear to be readily adaptable to vacuole fusion studies with a wide variety of other cell types, including mammalian leukocytes and macrophages.

We are very grateful for the valuable advice and generos-

ity of Dr. Robert J. Neff, who provided us with the use of his cell culture facilities throughout the course of this study.

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