

A MECHANISM FOR THE DESTRUCTION OF PINOSOMES IN CULTURED FIBROBLASTS

Piranhalysis

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ABSTRACT

The destruction of large pinosomes was examined with phase-contrast microscopy in cultured mouse fibroblasts. In areas of rapid pinosome breakdown, lysosomes were observed to repeatedly collide with pinosomes without fusing, tearing off small pieces until the pinosomes became smaller and denser. This segmentation of pinosomes by lysosomal collision has been named "piranhalysis."

KEY WORDS lysosomes · saltatory motion ·
pinocytosis · endocytosis · motility

When cultured mouse fibroblasts are observed under phase-contrast microscopy at high magnification, a surprising amount of intracytoplasmic motion of a nonrandom nature is visible, even without the aid of time-lapse recording. Although varying in degree from cell to cell, this intracytoplasmic organelle motion, referred to as "saltatory motion" (11), involves many different cytoplasmic organelles. The "vesicular" organelles visible with phase microscopy move with amazing speed, some traversing the entire width of a spread cell in less than a few seconds. Numerous papers have described the motion and activity of these structures (5, 3, 9, 12, 4, 2).

Among these vesicular organelles (excluding mitochondria), we have distinguished four general classes on the basis of microscope appearance: (a) phase-dense lysosomes which might be either "primary" or "secondary" and which freely move and collide with other structures, without necessarily fusing; (b) "grey" vesicles ($<0.3 \mu\text{m}$), with intermediate phase density, some of which seem to be

responsible for the immediate endocytic uptake of substances such as α_2 macroglobulin from medium (see reference 7); (c) the "clear" vesicles, which can be large ($>0.5 \mu\text{m}$) or small ($0.2\text{--}0.5 \mu\text{m}$), round or oblong, change shape rapidly, and freely fuse with one another or with the plasma membrane. These vary in number from cell to cell, being particularly evident in actively ruffling cells and in cells that have attached to substratum within the first day after subculturing by trypsin. Clear vesicles are macropinocytic vesicles (pinosomes) of the type originally described by Lewis (5), although some may form from fusion of multiple small micropinocytic vesicles. The motion and characteristics of these structures have been extensively studied (3-5, 9, 12 and others); (d) lipid vesicles, which are highly refractile, almost always round, move very little, and concentrate near the nucleus.

The collision of lysosomal organelles with pinosomes was originally described by Rose (12). His description of "microkinetospheres" colliding with pinosomes did not include any fragmentation of the pinosomes. The purpose of this paper is to show new evidence for fragmentation of pino-

some by repeated collisions with lysosomes that subdivide them into smaller pieces, a process we have named "piranhalysis."

MATERIALS AND METHODS

Swiss 3T3-4 mouse fibroblasts were obtained and propagated as previously described (15). For microscope observations, these cells were subcultured with 0.25% trypsin (Microbiological Associates, Walkersville, Md.) and planted in a Dvorak tissue culture chamber (Carl Zeiss, Inc., New York) in Dulbecco-Vogt's modified Eagle's medium with 10% calf serum (Colorado Serum Co., Denver, Colo.) and penicillin (50 U/ml) and streptomycin (50 µg/ml) (Flow Laboratories, Inc., Rockville, Md.). The culture chamber was slowly perfused with fresh medium (1 ml/24 h) by a syringe pump (Sage Instruments Div., Orion Research Inc., Cambridge, Mass.). The chamber was mounted on the stage of Zeiss RA upright or inverted microscopes enclosed in Lucite boxes in a 5% CO₂/95% air atmosphere, heated by a recirculation heating device that maintained the stage temperature at 37 ± 0.5°C. Observations were routinely made with a × 100 Neofluar (NA 1.3) phase 3 oil immersion objective (Zeiss) and a short working distance condenser (0.63) with a 15- or 60-W illuminator with GG3 (UV blocking) and Calflex (heat reflecting) filters (Zeiss). A closed circuit television camera (RCA TC 1030H, special low light level silicon intensifier target) was mounted on the normal Zeiss camera body, and this video signal was supplied to a Panasonic (model NV-8030) time-lapse video tape recorder and a Hitachi (model VM-904AU) monitor. The use of the low light level (<1 W with 15-W illuminator) camera allows illumination of such a small amount of light that the image is not visible by direct observation through the microscope eyepiece. Thus, light damage to the cells is minimized. The video image was recorded either at regular speed or at a 9/1 or 18/1 time lapse on 1/2 inch Hitachi video tape (R-176). Single-frame images were either photographed directly from the monitor with a Polaroid CU-5 camera or taken from 16-mm cine film produced from the video tape (Windsor Total Video, New York).

Transmission electron microscopy was performed by fixing cells grown on 35-mm² plastic dishes (BioQuest, BBL & Falcon Products, Cockeysville, Md.) with 2% glutaraldehyde (Tousimis Research Corp., Rockville, Md.) in 0.1 M cacodylate-phosphate-buffered saline (PBS) buffer for 20 min at 23°C. This was followed by fixation in 1.5% OsO₄ in PBS for 30 min at 4°C, ethanol dehydration, and *in situ* embedding in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi HU-12A electron microscope at 50 kV.

Acid phosphatase activity was studied with both light and electron microscopy by a modification of the method described by Novikoff (10). Cells were grown on cover slips, fixed in 1% glutaraldehyde in PBS for 10 min,

washed with 5% sucrose in H₂O for 1 h, and incubated in a reaction mixture containing 25 mg of cytidine monophosphate, 10 ml of 50 mM acetate buffer (pH 5), 12 ml of deionized H₂O, and 3 ml of 1% lead nitrate. This mixture was Millipore filtered (Millipore Corp., Bedford, Mass.), then incubated with the cells for 30 min at 37°C, and washed off with 5% sucrose in H₂O. Control samples were processed in the same manner except for the exclusion of the cytidine monophosphate from the mixture. For electron microscopy, the samples were then postfixed with OsO₄ in 0.1 M cacodylate and embedded in the normal manner. For light microscopy, the cells were further incubated in 1% ammonium sulfide in H₂O for 30 min at 23°C and then photographed using phase optics and Kodak Tri-X film.

RESULTS

Fig. 1 shows two sequences taken from a video tape record of the formation of large, clear vesicles in the cytoplasm of spread Swiss 3T3 cells. These large, clear vesicles form through the engulfment of medium during ruffling or pseudopod extension as originally described by Lewis (5). This process occurs both at leading lamellae and on the free surface of these fibroblasts which occasionally show ruffling activity independent of lamellar extension. In the 30-s frame (Fig. 1A), a ruffle has begun to form on the upper surface (arrows). At 86 s, the field is focused on the surface and the ruffle has swept over the upper right part of the field and begun to re-incorporate into the upper cell membrane (arrows). Soon after this event (150 s), a large, clear vesicle appears in this region (asterisk) under the ruffle, and other vesicles form in the ruffle itself. One of these (small arrow) is destined to be a small vesicle after being pushed into the cytoplasm (at 165 s), and another (large arrow) is a fusion product of two separate vesicles (seen at 150 s) which enter the cytoplasm (at 195 s) as a single large, clear vesicle. The surface ruffle has completely disappeared at 195 s, and the vesicles are pushed and torn by surrounding lysosomes (357 s) until only smaller and darker remnants remain (627 s).

Fig. 1B shows a region with numerous clear vesicles already apparent (*cv*). The destruction of these vesicles seems to be much slower than that of those seen in Fig. 1A. At 180 s, a ruffle begins to form on the upper surface (arrows) which, when focused on the surface region at 200 s, shows multiple large vesicles contained within. Two of these large structures (asterisks) fuse to form a single large vesicle at 290 s. As the ruffle is

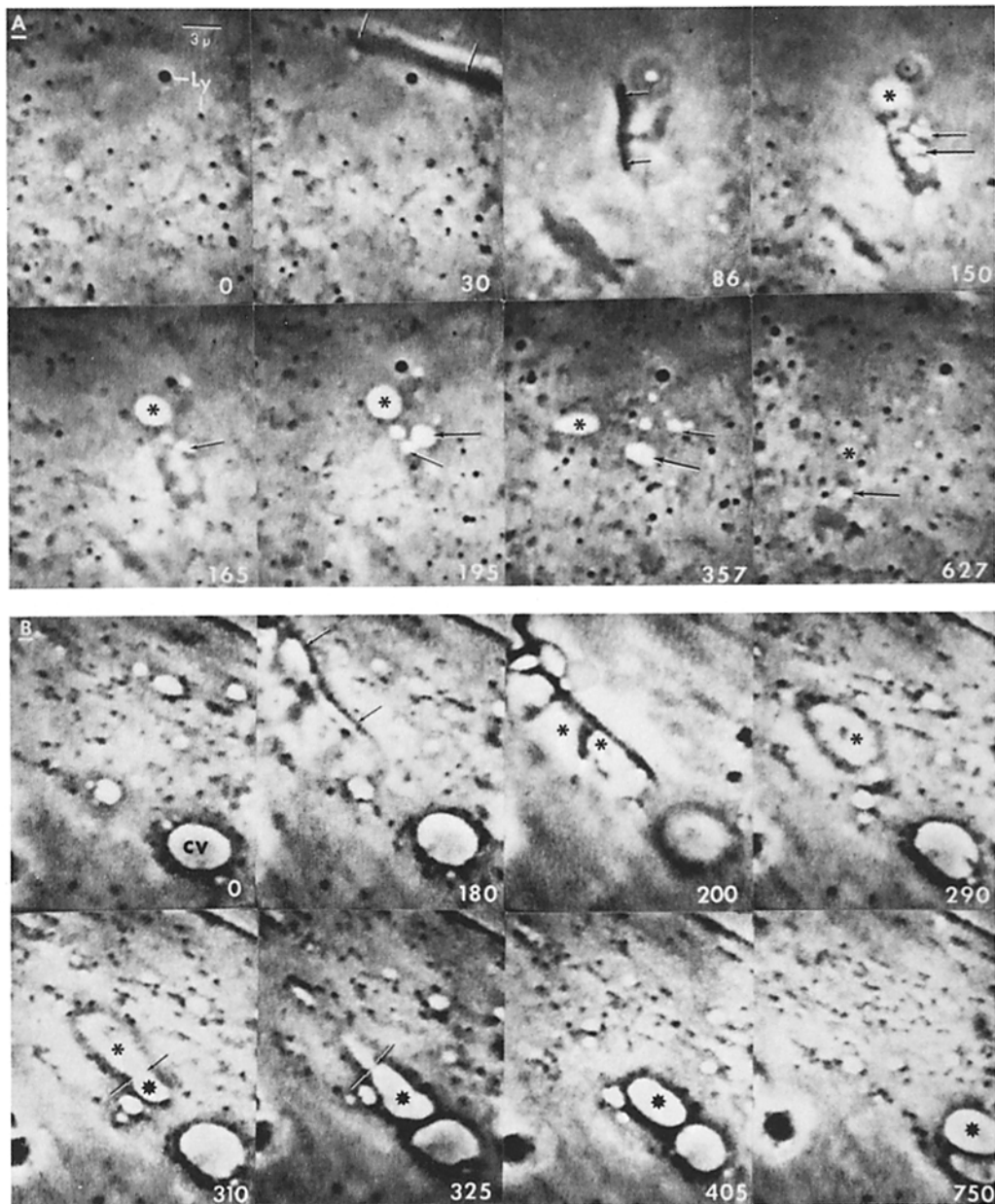


FIGURE 1 The formation of clear vesicles. (*A* and *B*) This shows multiple video frames of a region of cytoplasm in a spread Swiss 3T3 cell that is undergoing frequent ruffling on the upper surface. Numerous phase-dense lysosomes (*Ly*) populate this region, varying from ~ 0.2 to $1.0 \mu\text{m}$ in size. *CV*, Clear vesicle. The numbers on the corner of each frame represent the time (in seconds) after the beginning of each sequence. (See text for explanation of sequences.) (Video phase contrast.) $\times 1,680$.

resorbed into the cell, this large vesicle is injected into the cytoplasm through an apparent restriction (arrows at 310 s and 325 s). As it enters the cytoplasm (star), it assumes an oblong shape (405

s) and is slightly displaced with little or no destruction at 750 s.

The process by which pinosomes are degraded is most evident in actively ruffling cells, many days

after subculturing, in which a newly formed vesicle is destroyed within a short time. Fig. 2 shows a sequence of frames from a recording of a clear vesicle being degraded. Multiple lysosomes collide with the vesicle, distorting its outline and often even stretching the vesicle in a long, thin extension which then breaks off. After multiple collisions, portions of the vesicle are broken off and carried away by these lysosomes, the vesicle often rapidly decreasing in size and becoming more phase-dense. Eventually, the entire vesicle has been "cleaned up" by this process. Since the film of this process is reminiscent of a feeding frenzy, the name piranhalysis has been chosen to describe what appears to be voracious destruction by these lysosomes.

The ability to see this interaction requires certain conditions. First, the area of the cell must be exceedingly thin (1–1.5 μm) to allow sufficient optical clarity. This was the reason why we chose such thin, flat cells to demonstrate this phenomenon. Unfortunately, to make these observations also requires the presence of surface ruffle activity which is relatively infrequent in flat cells. As a result, finding an appropriate field requires considerable searching. Some cells also seem to have less active cytoplasmic saltatory activity, and other cells have areas of their cytoplasm in which lysosomes segregate from the pinosomes. Thus, this phenomenon is not necessarily immediately obvious. We have observed this type of interaction on at least 10 occasions over the course of 3 days of observation. Considering the optical and cell morphology limitations required to observe this interaction, this could not be called a rare event. However, it is not at all clear that this is the most common form of lysosome-pinosome interaction. It is presented here only as an alternative to the concept of rapid and complete fusion of lysosomes with other vesicles.

Many of the vesicles visualized by phase microscopy have obvious electron microscope counterparts:

(a) Lipid vesicles, which appear highly refractile with phase contrast, appear intermediate in density and uniform after OsO_4 fixation in transmission electron microscope images.

(b) Phase-dense lysosomal structures seemed to correspond to primary and secondary lysosomes. The term primary is used here only in a morphologic sense to denote a dense, uniform, membrane-limited, vesicular organelle. Secondary is used only to imply the appearance of these organ-

elles after fusion with other vesicles. These secondary lysosomal structures often show a mixture of dense and less dense contents. These terms are not intended as precise demarcations of lysosomal classes or specific histochemical criteria (1). However, to clarify the exact nature of the "lysosomal" vesicles, and to insure that the organelles we observed colliding with pinosomes were indeed lysosomal by other criteria, we studied their acid phosphatase cytochemistry as was done in earlier studies (2) and their electron microscope morphology.

Fig. 3 shows the light microscope appearance of an area in these cells in which pinosomes are interacting with phase-dense vesicular lysosomes. As can be seen in this figure, virtually all of the phase-dense lysosomal dense bodies contain acid phosphatase activity. At the ultrastructural level, the same finding was readily apparent (Fig. 4A).

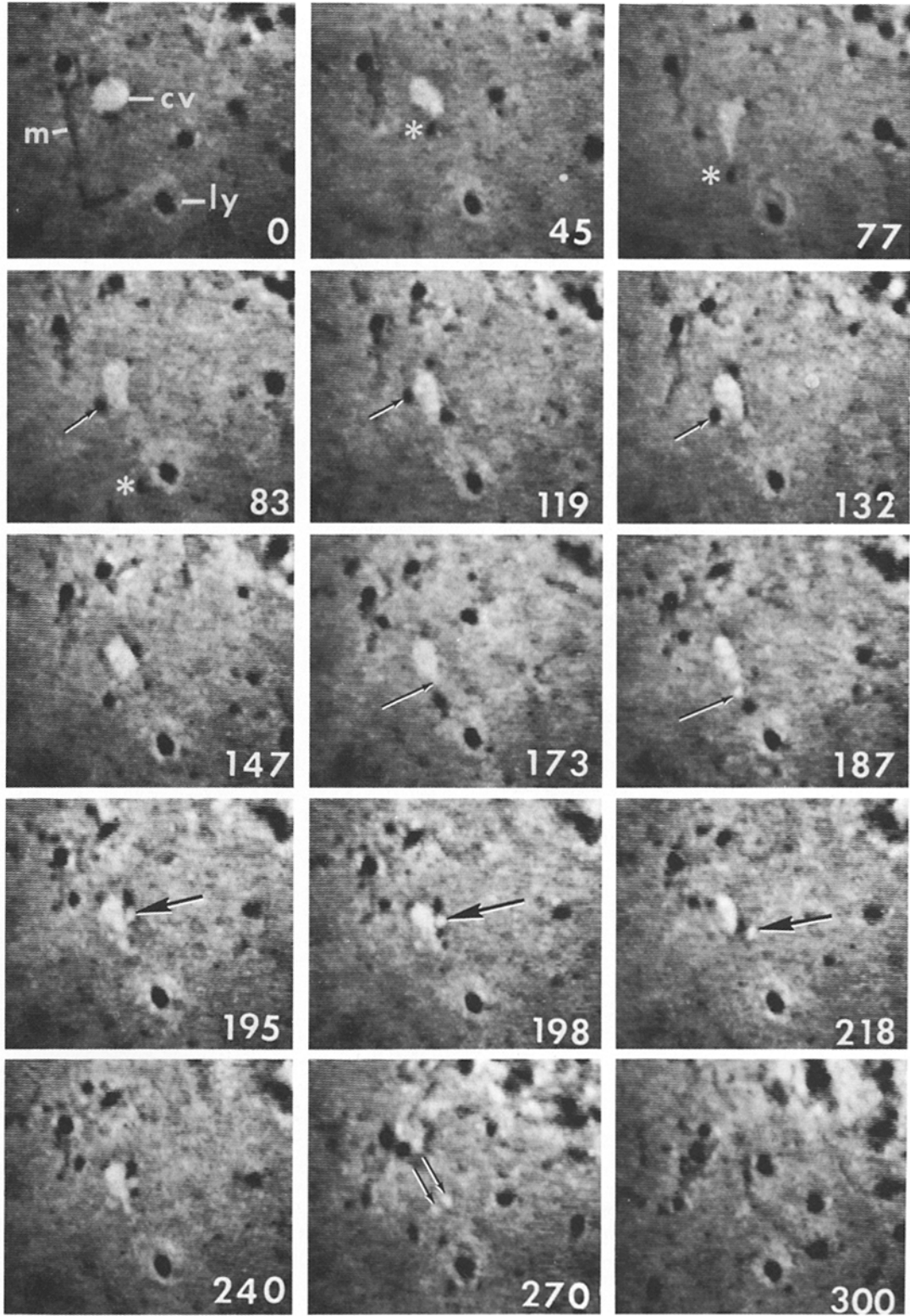
The ultrastructural morphology of typical examples of these lysosomal structures is also shown in Fig. 4B and C. As can be seen in these figures, these organelles have a homogeneous dense internal content with a clear zone under the limiting membrane. Thus, by these morphologic and cytochemical criteria, these organelles appear to be lysosomes.

(c) Clear vesicles, since they contain only proteins from culture medium at a low concentration, appear virtually clear in the electron microscope. Some clear vesicles appear to have undergone repeated fusion with other clear vesicles (based on their multilobulated shape), have decreased in size, and have a more dense internal content in agreement with previous descriptions (3, 5) (results not shown).

(d) Grey vesicles are still difficult to correlate, since it is possible that they correspond to "coated" (8) vesicles and later to uncoated vesicles of an intermediate density, somewhat similar to the internal density of the endoplasmic reticulum cisternae. Indeed, it is not clear that some rough endoplasmic reticulum may not have a separated vesicular form that could correspond to some of the grey vesicles observed with phase microscopy. The exact nature of these grey vesicles, some of which often contain α_2 macroglobulin selectively taken up from the medium (see reference 7), awaits specific labeling of these components with ultrastructural markers.

DISCUSSION

The uptake of components from outside the cell is



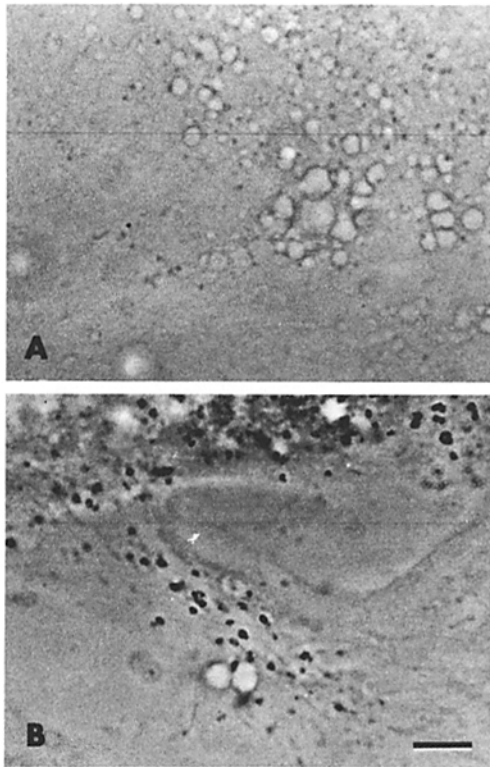


FIGURE 3 Light microscope appearance of acid phosphatase cytochemistry of lysosomal organelles interacting with pinosomes. Acid phosphatase was detected by the method of Novikoff (10) as described in Materials and Methods. Areas of Swiss 3T3 cells with visible lysosome-pinosome interactions were photographed after cytochemical reaction with phase-contrast optics to enhance photographic contrast either without (A) or with (B) the enzymatic substrate (CMP). Bar, 5 μm . $\times 1,400$.

a common occurrence *in vivo* (13) and is particularly striking in such instances as macrophage engulfment of bacteria or erythrocytes (6), or the

uptake of thyroglobulin from the thyroid follicular lumen (16). These endocytic processes involve the extension of pseudopods around the engulfed object, fusion of opposing membranes to form an intracytoplasmic vacuole, and resorption deeper into the cytoplasm of this phagocytic vesicle. When a ruffling edge folds back onto the plasma membrane, however, no specific object is being engulfed, only culture medium. Thus, this macropinocytic process results in large vesicles with a low density content compared to the rest of the cytoplasm. These vesicles are pushed around in the cytoplasm by lysosomal motility and other vesicular motion, and often will spontaneously fuse if they collide with similar vesicles as described by Lewis (5). Lysosomes seem to attach readily to these vesicles as described by Rose (12), but rather than the expected rapid fusion with the vesicle on contact (14), the lysosomes only attach to, and have not been observed to directly fuse with a large vesicle. The only specific interaction other than attachment that is evident is the fragmentation process described in this paper.

It is possible that piranhalysis allows breakdown of the large vesicle into small enough units to allow lysosomal fusion, but this has not yet been observed. Another possibility is that macropinocytic vesicles continue to undergo micropinocytosis as they did when they were still part of the external plasma membrane. Lysosomes could then simply pick up these smaller pinosomes to which they attach on collision. The lysosomes, rather than the pinosomes, seem to be the motile elements in these interactions. The mechanism of this lysosomal saltatory motion (11) and the exact nature of the lysosome-pinosome interaction remains to be elucidated.

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FIGURE 2 The process of piranhalysis in the destruction of clear vesicles by lysosomes. This figure is a collection of still frame video images from a single region of cytoplasm in which clear vesicles were being rapidly degraded. At time 0, a clear vesicle (*cv*), numerous lysosomes (*ly*), and a mitochondrion (*m*) are visible. At 45 s, a single lysosome (asterisk) attaches to the clear vesicle and begins to migrate downward. By 77 s, it has greatly distorted the shape of the vesicle, and by 83 s it has pulled away, taking with it a small piece of the vesicle. Another lysosome (at 83 s, arrow) attaches and pulls on the vesicle (at 119 s), apparently breaking off multiple pieces that remain attached to it (at 132 s). The same lysosome returns to reattach (at 173 s) and tears away an even larger piece (arrow) (at 187 s). These multiple small pieces rapidly diminish in size and become invisible within a few seconds. Another lysosome attaches (at 195 s) and seems to rend another piece from the vesicle (arrow) which is finally fully separated at 218 s (arrow). Further collisions occur and small pieces are lost, until the visible vesicle remnants are two smaller fragments (at 270 s, arrows) which gradually disappear (300 s). This tearing collision of lysosomal structures with clear vesicles is the process we refer to as piranhalysis. (Video phase contrast.) $\times 3,100$.

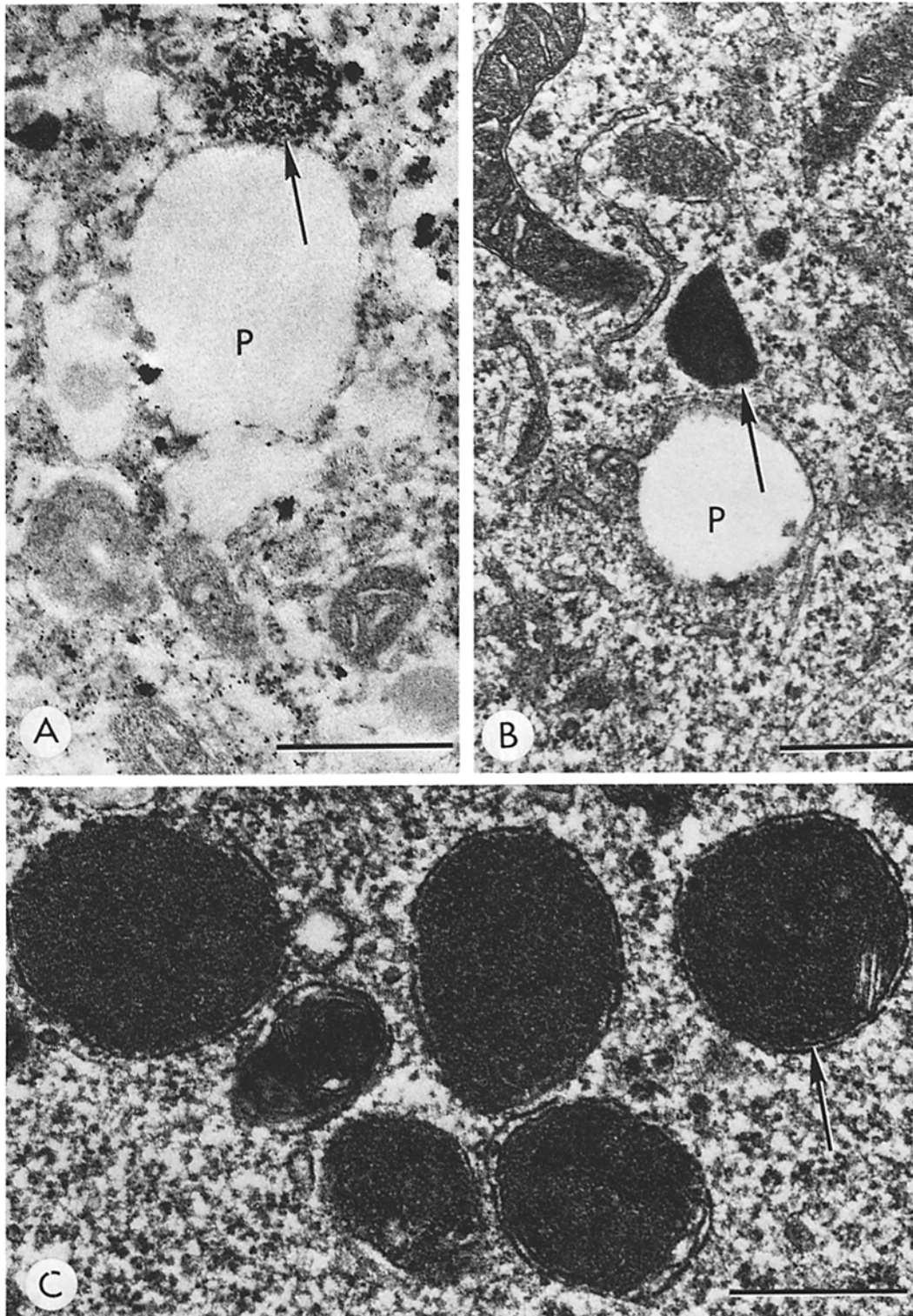


FIGURE 4 Ultrastructural acid phosphatase cytochemistry and morphologic appearance of lysosomal organelles. (A) Acid phosphatase cytochemistry was performed as described in Materials and Methods. Dense reaction product is visible associated with a lysosomal vesicle (arrow) attached to a pinosome (P). Fig. 4B and C represent the morphologic appearance of these lysosomal organelles (arrow) in proximity to a pinosome (P) in Fig. 4B and in an example of the characteristic electron-transparent rim (arrow) under the limiting membrane in Fig. 4C. (A) Unstained, (B and C) uranyl acetate-lead citrate-stained. Bars, 0.5 μm . (A) $\times 53,000$; (B) $\times 41,000$; (C) $\times 53,000$.

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