A NOVEL MODEL FOR FLUID SECRETION BY THE TRYPANOSOMATID CONTRACTILE VACUOLE APPARATUS

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ABSTRACT

We have studied fluid secretion by the contractile vacuole apparatus of the trypanosomatid flagellate *Leptomonas collosoma* with thin sections and freeze-fracture replicas of cells stabilized by ultrarapid freezing without prior fixation or cryoprotection. The ultrarapid freezing has revealed membrane specializations related to fluid segregation and transport as well as membrane rearrangements which may accompany water expulsion at systole. This osmoregulatory apparatus consists of the spongiome, the contractile vacuole, and the fluid discharge site. The coated tubules of the spongiome converge on the contractile vacuole from all directions. These 60- to 70-nm tubules contain characteristic double rows of 11-nm intramembrane particles in a helical configuration which fracture predominantly with the E face. Short double rows of similar particles are also frequently found on both faces of the contractile vacuole itself, in addition to many smaller particles on the P face. The spongiome tubules fuse with the vacuole during the filling stage of each cycle and then detach before secretion. The contractile vacuole membrane is permanently attached to the plasma membrane of the flagellar pocket by a dense adhesion plaque. In some ultrarapidly frozen cells, 20- to 40-nm perforations can be visualized within the plaque and the adjacent membranes during the presumptive time of discharge. The formation of the plaque perforations and the membrane channels occurs without fusion of the vacuole and the plasma membrane and does not require extracellular calcium. On the basis of our results, we have developed a model for water secretion which suggests that the adhesion plaque may induce pore formation in the adjoining lipid bilayers, thereby allowing bulk expulsion of the fluid.

KEY WORDS secretion · contractile vacuole · freeze-fracture · membrane fusion · trypanosomatid

The study of the membrane events during secretion is significant for our understanding of broad physiological problems as well as for molecular models of membrane behavior. Fluid secretion in protozoa offers several promising areas for such examination. Our research on the ultrastructure of the contractile vacuole (CV) apparatus of the trypanosome-like organism *Leptomonas collosoma* has concentrated on two main questions: the nature of the membrane systems involved with segregation and transport of fluid, and the transient membrane rearrangements which create a channel for water

expulsion during systole.

Because some of the events of fluid secretion are potentially very susceptible to osmotic variation and others (such as systole) may involve extremely rapid membrane changes, we felt that it was necessary to augment the traditional techniques of thin-section and freeze-fracture electron microscopy with ultrarapid freezing of unfixed cells without cryoprotectants. By using copper sandwiches, spray freezing, and jet freezing with liquid propane, we have been able to examine both thin sections and freeze-fracture replicas of cells quickly frozen in their natural state.

In most previous studies on trypanosomatid flagellates, the CV apparatus has been treated very superficially (4, 5, 19, 27, 28, 30). The only exception pertains to a thin-section study of *Crithidia* by Brooker (3), who noted that the 70-nm spongiome tubules, where initial fluid segregation is presumed to occur, are coated with 10-nm spines on their cytoplasmic surface. However, there have so far been no reports on cyclic variations in the spongiome-CV association or any specialized structures involved with fluid expulsion in trypanosomatids. For other types of protozoa, Kitching (12, 13) has reviewed CV physiology and McKanna (15) has analyzed the literature on CV ultrastructure. McKanna (15–18) has also contributed to the fine-structure studies on CVs and has emphasized that small-diameter tubules or vesicles coated with helically arranged pegs on the cytoplasmic surface of the membrane form the initial fluid segregation organelles in all species examined.

Freeze-fracture studies to examine CV membranes involved in fluid segregation and release have been very rare. Hausmann and Allen (9) and Allen (1) have described the intramembrane particle distribution on the fluid segregation organelles, spongiome, collection canals, and the pore wall of the complex excretory apparatus of *Paramecium*, concentrating on the membrane-microtubule associations. They were not able to present any information on the vacuole itself and its contraction during systole. Weiss et al. (31) have demonstrated a specialized CV-plasma membrane junction in *Chlamydomonas*, but they failed to find any membrane openings for fluid expulsion.

Theories about membrane reorganization for water expulsion at systole must rely on similarities with respect to other types of secretion. Satir (25) has proposed two general models, involving either membrane gate release, where tiny hydrophilic channels pair up in the two adjacent membranes,

or else membrane fusion followed by bulk release of the contents of the secretory vesicles. Most secretory systems so far studied, including the *Paramecium* CV (16), are thought to involve fusion of the vesicle with the plasma membrane (10, 22–24, 26). Frequently, these fusion events are dependent on extracellular calcium (7).

We have used cultured *Leptomonas* cells, originally from water strider hindgut (29), to extend the studies of the trypanosomatid fluid secretion apparatus. The basic ultrastructure and plasma membrane specializations of this flagellate have been presented in a previous publication (14). In this report, we concentrate on the membranes of the CV apparatus and describe intramembrane specializations on the fluid-segregating spongiome as well as on the CV itself. More importantly, however, we examine the membrane rearrangements during water expulsion and present evidence to support the idea that fluid secretion may occur without membrane fusion or assembly of tiny aligned hydrophilic channels as in membrane gate release. On the basis of our findings, we have developed a new model for fluid secretion which involves an adhesion plaque located between the CV and plasma membranes to induce pore formation in the two parallel lipid bilayers.

MATERIALS AND METHODS

A culture of *L. collosoma* was the gift of Dr. F. G. Wallace, Department of Zoology, University of Minnesota. Cells were grown in proteose-peptone medium as described by Hunt and Ellar (11). Some cells were treated with a 50 mM Tris-HCl (pH 7.8) buffer containing up to 40 mM EGTA for various times.

Two basic methods were used for thin-section electron microscopy.

Glutaraldehyde fixation: After 1–2 h in 2% glutaraldehyde, cells were postfixed for 1 h in 1% OsO_4. All fixations and washes were carried out with 0.16 M phosphate buffer, pH 7.4. Cells were dehydrated in an acetone series or with acidified dimethoxypropane (21).

Freeze-substitution: Cells suspended in growth medium were sprayed into liquid propane with a Balzers Spray-Freeze apparatus (Balzers Corp., Nashua, N. H.). The frozen droplets were infiltrated with 2–5% OsO_4 in dry acetone at −90°C for 1–2 h, rewarmed, and washed twice in dry acetone.

All cells prepared for thin sectioning were embedded in Epon-Araldite and sectioned with a Porter-Blum MT-1 microtome (DuPont Instruments-Sorvall, DuPont Co., Newton, Conn.). Sections were stained with uranyl acetate and lead citrate.

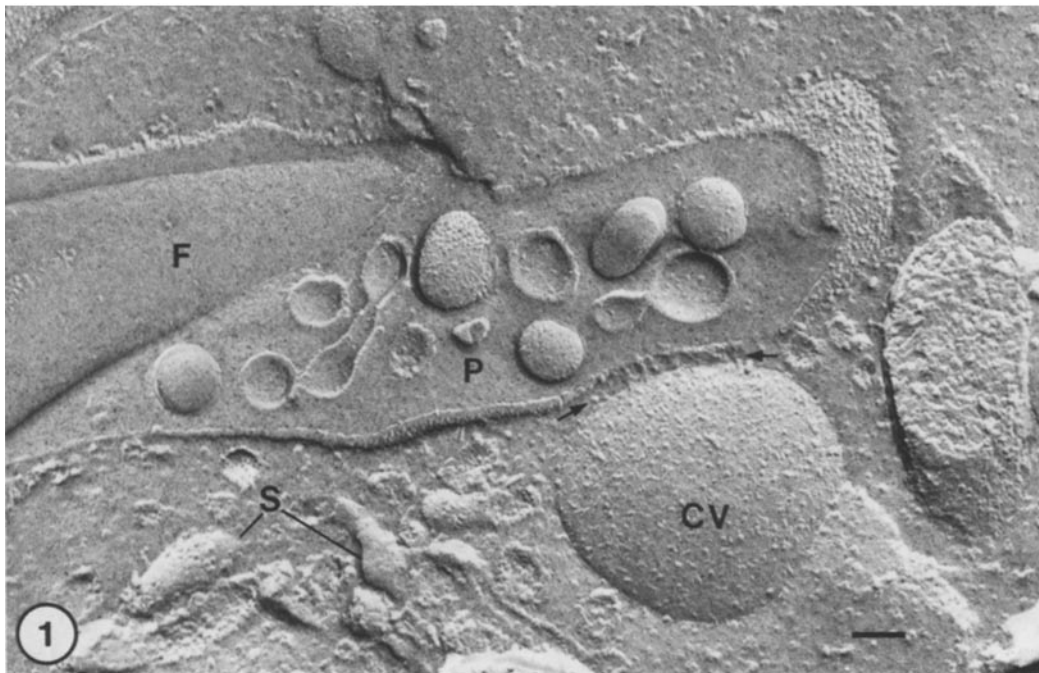Cells were either glycerinated or ultrarapidly frozen before freeze-fracturing.

FIGURE 1 Freeze-fractured *Leptomonas* cell stabilized by jet-freezing showing the components of the CV apparatus. The contractile vacuole (*CV*) adjoins the flagellar pocket (*P*) by the fibrillar adhesion plaque (arrows) while spongiome (*S*) tubules are seen nearby. This freezing method produces excellent preservation of the membranes as evidenced by their smooth, turgid appearance. *F*, flagellum. × 71,000. Bars in all figures, 0.1 μm.

Glycerination: Unfixed cells suspended in growth medium were slowly infiltrated with 25–35% glycerol over 2.5 h. The pelleted cells were frozen in liquid Freon 12.

Ultrarapid freezing: Cells were pelleted from growth medium with no added cryoprotectant. Some were spray frozen as for the freeze-substitution experiments and then mounted on gold specimen holders as described by Bachmann and Schmitt-Fumian (2). Others were placed in a very thin layer between two copper or gold disks (8), and these "sandwiches" were either plunged into liquid propane by hand or else frozen with two pressurized streams of liquid propane from a jet freezer (20).[1]

A Balzers BA360 freeze-etch apparatus was used to fracture glycerinated, spray-frozen, or copper sandwich-frozen cells at −108°C and to prepare a platinum-carbon replica of the exposed area. Jet-frozen samples were fractured in a Balzers double replica device at −120° or −130°C. All replicas were cleaned in bleach and chromic acid.

Thin sections and replicas were observed with a Philips EM200.

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[1] Müller, M., N. Meister, and H. Moor. 1979. Freezing in a propane jet and its application in freeze-fracturing. Manuscript submitted for publication.

RESULTS

Leptomonas generally has one CV apparatus located at the anterior end of each cell (Fig. 1). It lies adjacent to the flagellar pocket, into which it empties, and close to a novel membrane lattice (to be described in a subsequent publication) and the Golgi complex. The CV itself is filled from coated tubules of the spongiome. The vacuole expels its contents at a specialized discharge site where it adheres to the plasma membrane of the flagellar pocket. The spongiome, vacuole, and discharge site will each be described in turn.

Spongiome

The spongiome of the CV apparatus consists of 60- to 70-nm tubules which surround the vacuole and converge upon it (Figs. 1, and 2 a and b). They are sometimes curved or branched. At their distal ends, they can be continuous with the CV (Fig. 2 b).

The tubule membranes may be recognized by a coat of 16 × 5.5-nm pegs projecting from their cytoplasmic surface. There is some suggestion of

a fuzzy coat on the lumenal surface as well (Fig. 2a). Freeze-fractured tubule membranes show that the lumenal E face is frequently distinguished by 11 ± 2-nm particles arranged in apparently helical double rows on an otherwise smooth background (Fig. 3a). Because some replicas may have been inverted, we cannot be certain of the handedness of this helix. The protoplasmic P face, on the other hand, is covered with randomly arranged 6- to 13-nm intramembrane particles with only a faint
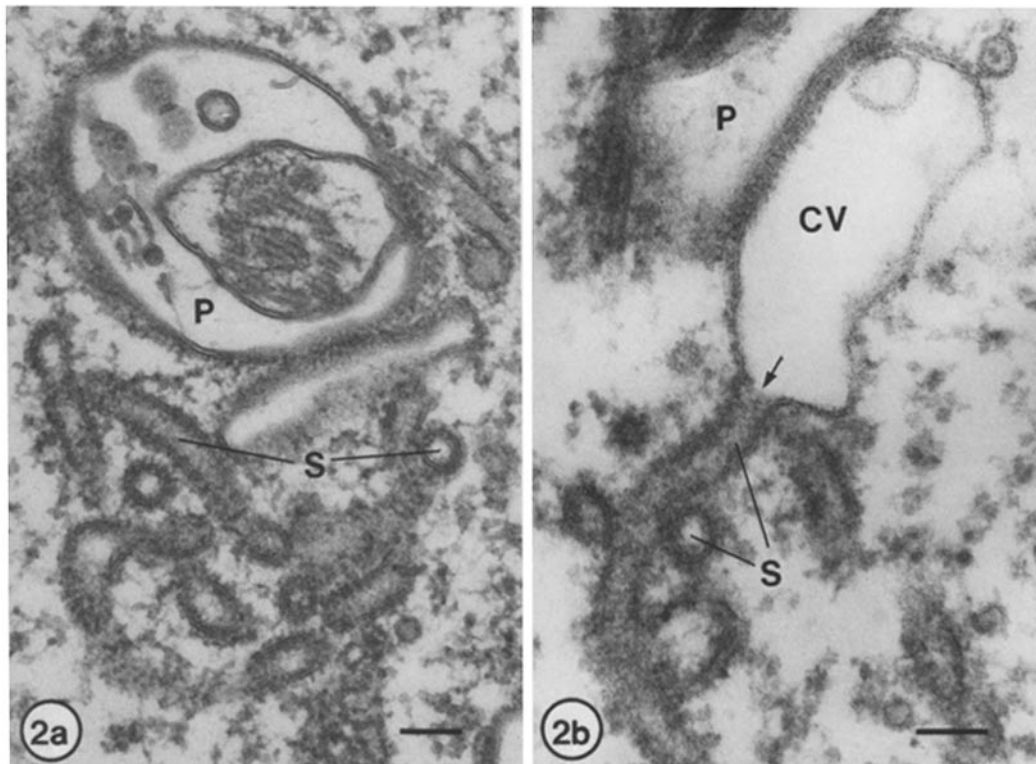

FIGURE 2 (a) Coated 60- to 70-nm tubules of the spongiome (S) are seen in cross and longitudinal section next to the flagellar pocket (P). Note the fuzzy coat on the lumenal surface of the tubules. Freeze-substituted. × 77,000. (b) This contractile vacuole (CV) near the flagellar pocket (P) demonstrates continuity (at arrow) with a tubule of the spongiome (S). Glutaraldehyde fixed. × 90,000.
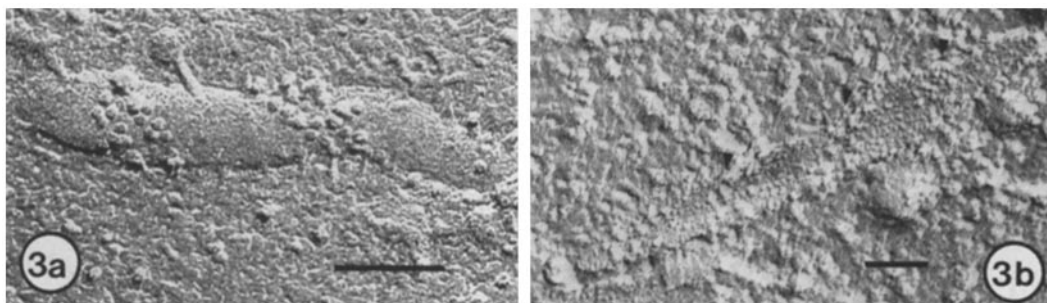

FIGURE 3 (a) This micrograph shows an E face view of a freeze-fractured spongiome tubule. Note the characteristic, widely spaced, diagonal double rows of 11-nm particles. Glycerinated. × 145,000. (b) The P face of this spongiome tubule is generally covered with randomly arranged particles. A suggestion of additional diagonally arrayed particles and pits may be seen at the arrowheads. Frozen without cryoprotectant. × 84,000.

suggestion of the helical pattern (Fig. 3 b). These particle arrangements can be seen after any of the freezing methods used.

Contractile Vacuole

The *Leptomonas* CV is always found immediately adjacent to the plasma membrane of the flagellar pocket (Figs. 1, 2 b, 4 a–f, 5 a–d, 6 a–f, and 7). In rare cases, a second vacuole is nearby. The shape and size of the CVs seem to vary with the stage of the cycle (Fig. 4). Thus, the CVs can appear rather small (Fig. 4 a) and/or flattened (Fig. 4 b), irregular with many spongiome connections (Fig. 4 c), or large and spherical (Figs. 4 d–f, and 6 a–f). The expanded ones can reach 0.6–0.9 μm in diameter and bulge into the lumen of the pocket. Occasionally, flocullant material is seen inside the vacuole.

Freeze-fractured CV membranes are remarkable in that they frequently contain 10 ± 3-nm particles arranged in distinct patches. These particles often occur in regular arrays and may be seen on either the P- or E-fracture face, regardless of the freezing method (Fig. 4 d and e). The patches are unevenly distributed in the CV membrane and are not visible at all on a minority of fractured vacuoles (Figs. 1 and 4 f). The remaining smaller particles are randomly arranged within the CV membrane, with more of them adhering to the P face. In thin sections the CV sometimes demonstrates an occasional patch of fuzzy material of the lumenal surface (Fig. 7), but the cytoplasmic surface appears smooth, unlike that of the spongiome tubules.

Many vacuole membranes show continuity with those of the spongiome tubules. There seems to be an attachment-detachment cycle of the spongiome tubules to the vacuole membranes because either many connections (Fig. 4 c) or none are seen. When none are seen, the vacuole usually appears in a highly inflated state (Figs. 4 e and f, and 6).

Fluid Discharge Site

The CV is attached to the side of the flagellar pocket by a specialized region which we have termed the adhesion plaque (Figs. 1, 4 a–c, 5 a–d, and 7). In that area the two membranes follow a parallel course ~25 nm apart. The space between them is filled by a plaque of dense, possibly fibrillar material ~300 nm in diameter. We have never seen a CV membrane in direct physical contact with the flagellar pocket membrane; the plaque of
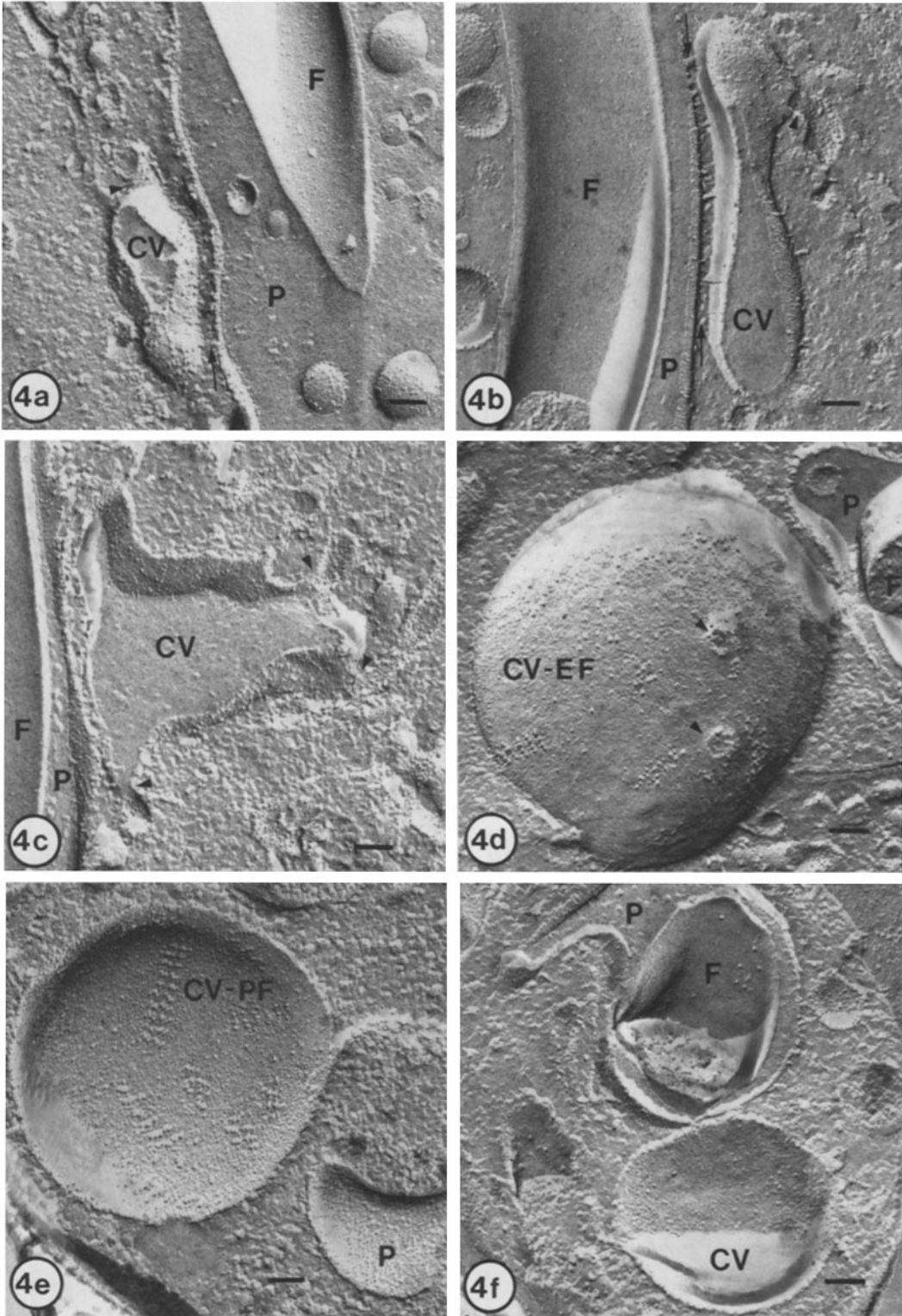
dense material always intervenes, whether the vacuole is collapsed, filling, or large and spherical. However, the appearance of the plaque material varies (Figs. 5 a and c, and 7) and may reflect changes in its structure with different stages of the CV cycle. Freeze-fracture through the regions of the CV membranes overlying the plaque shows no patches or spongiome tubule connections (Fig. 6 a–f). In some fully expanded vacuoles, the junctional membrane region can be recognized because of a series of deformations of the CV membrane (Fig. 6 a, b, and d). The density of particles seems to be reduced in this puckered area. In addition, we have on several occasions observed a small number of 20- to 40-nm round perforations, presumptive membrane pores, in the midst of these membrane perturbations after cells have been ultrarapidly frozen without cryoprotectants (Fig. 6 c–f). Fig. 7 shows a thin section through a similar area. Although a channel has been created between the lumen of the expanded CV and the pocket, through both the CV and plasma membranes (Figs. 6 f and 7), these membranes are still held apart by the adhesion plaque and do not actually fuse with each other.

Although the freeze-fractured pocket plasma membrane contains several distinct kinds of particle arrays (14), none of them seems related to the CV adhesion plaque or discharge site. We have never seen anything like a rosette (26) on either junctional membrane (Figs. 5 d and 6 c). It is impossible to precisely characterize the plasma membrane overlying the CV because there are no landmarks to show where the CV lies beneath the membrane. The interpretation is further complicated by frequent membrane openings due to the extensive exo- and endocytosis occurring at the pocket plasma membrane (3).

The involvement of extracellular calcium in CV functioning was studied by placing washed cells in various low-calcium solutions. In no case did we see an effect on CV contraction. Our most extreme treatment of 40 mM EGTA in Tris buffer should have removed all free calcium. Yet, after 27 h in this solution, it was still possible to watch vacuoles contracting with the light microscope. Some cells continued to swim and maintained their normal elongated shape after 48 h in this calcium-free buffer.

DISCUSSION

In this report we confirm and extend previous observations on trypanosomatid CVs and also con-

tribute to the sparse literature on freeze-fractured CV membranes. Our work on *L. collosoma* has produced some intriguing results, for two main reasons. First, our ultrarapid freezing techniques have preserved transient events which otherwise might have been disturbed by fixatives, cryoprotectants, or ice crystal damage. Secondly, *Leptomonas* is such a tiny cell (10 × 2 μm) that the CV apparatus is not hidden among large volumes of other organelles and membranes, as in complex ciliates. Thus, we can easily examine hundreds of vacuoles for rare events such as the rapid membrane alterations occurring at systole. Our results also include observations on membrane specialization of the spongiome and vacuole. Because the new information on membrane events during systole presented in this publication differs from that on the membrane fusion seen in most other types of secretion (10, 22–24, 26), we have developed a new model for fluid secretion which does not depend on membrane fusion, yet still produces open pores for bulk water expulsion.

A general diagram of the *Leptomonas* CV apparatus is presented in Fig. 8. The vacuole is attached to the plasma membrane of the pocket by the adhesion plaque throughout the cycle. Spongiome tubules converge on the CV from all directions. In most cells, the membrane lattice and the Golgi apparatus are located between the nucleus and the CV. The cyclic changes in the CV are diagrammed in Fig. 9. During diastole, the spongiome tubules fuse to a flattened CV which then swells as fluid accumulates. After the vacuole has reached its maximum size, the tubules detach. During systole, pores form between the lumens of the CV and the pocket. The vacuole contracts, shrinking again to a flattened sac as fluid is released. The pores then close, and the cycle repeats. Extracellular calcium does not seem to be required for the creation of these pores because vacuoles contract normally in the presence of 40 mM EGTA.

Systole

An admittedly speculative model of the membrane events at the discharge site is presented in Fig. 10. The discharge site is marked by a 25-nm thick adhesion plaque which might play an active role in creating small pores between the CV and the lumen of the pocket. One possible mechanism would be a conformational change in the plaque material, creating small perforations so that the dense junctional material resembles a sieve (see Figs. 5a and b, and 7). Contraction of the vacuole might then rupture the destabilized lipid bilayers and allow the release of the fluid. At this point the adhesion plaque would presumably prevent fluid from leaking into the surrounding cytoplasm and also would stabilize the adjacent membranes against the forces occurring during fluid expulsion. At the end of systole, the plaque would return to its original state, the membranes would reseal themselves, and the flattened vacuole would be ready for the next cycle of filling and contraction.

It is possible that systole is similar for the *Chlamydomonas* CV. Weiss et al. (31) published pictures of a dense plaque and puckered membrane similar to those seen in *Leptomonas*. The fact that they never observed membrane pores in their freeze-fractured material provides only negative evidence for a membrane gate release for the fluid. We would like to suggest that they were simply unable to freeze their cells fast enough with conventional methods to preserve the small-diameter perforations of the type observed in the present study (a possibility which they also discussed). Thus, we believe that our ultrarapid freezing techniques are responsible for allowing us to extend the CV story one step further. It is unlikely that the membrane perforations we observe are artifacts because the

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FIGURE 4 (*a–f*) These micrographs suggest stages during diastole of the CV cycle. At the beginning of the cycle, the small (Fig. 4*a*) or collapsed (Fig. 4*b*) vacuole (*CV*) is connected to spongiome tubules (arrowheads) and joined to the pocket (*P*) membrane by material of the adhesion plaque (arrows). As the vacuole fills with fluid from the spongiome, it takes on an irregular shape (Fig. 4*c*) with many spongiome connections and eventually expands to form a sphere (Fig. 4*d–f*). Cross-fractured connections to the spongiome can still be seen in Fig. 4*d* (arrowheads) but are absent in later stages (Fig. 4*e*). Several patches of 10-nm particles in arrays that resemble the double rows of particles seen on the spongiome tubules (compare with Fig. 3*a*) are found on both the E face (Fig. 4*d*) and the P face (Fig. 4*e*) of many vacuoles. The vacuole membrane adjacent to the adhesion plaque and overlying the pocket plasma membrane is generally undifferentiated (except for a possible reduction in particle number) during diastole (Fig. 4*f*). *F*, flagellum. All cells frozen without cryoprotectant. All × 61,000.
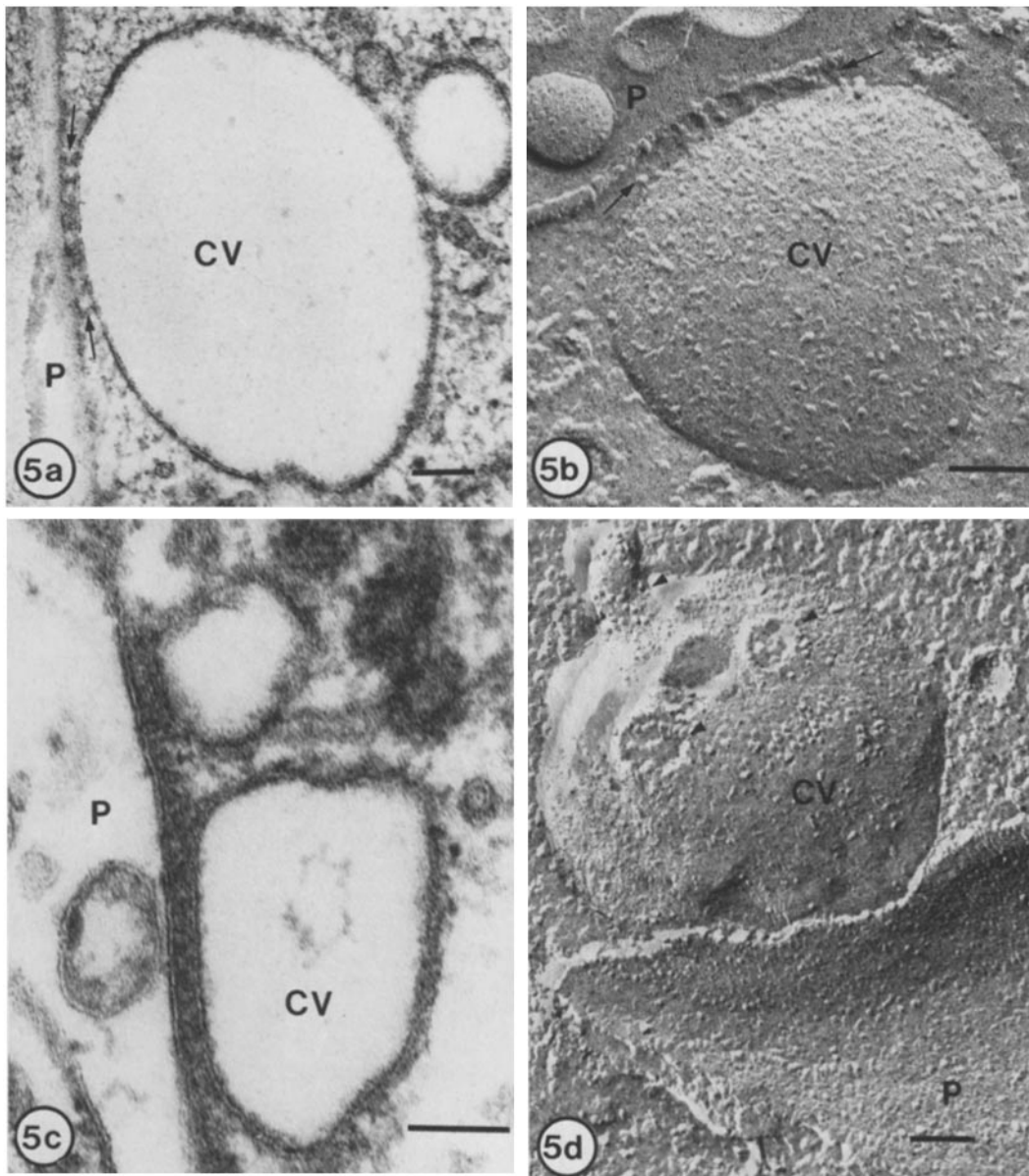
FIGURE 5 (a–d) The specialized adhesion plaque between the CV and the pocket (P). Dense, striated material fills a 25-nm gap between the two parallel membranes in both thin-sectioned (Fig. 5a and c) and freeze-fractured (Fig. 5b) cells. The plaques in both Fig. 5a and b (enlarged from Fig. 1) appear to contain several perforations (between the arrows). Fig. 5d shows that the pocket membrane adjacent to the CV in the plaque region is quite undifferentiated. Arrowheads mark spongiome tubule connections. (a) Freeze-substituted. × 83,000. (b) Frozen without cryoprotectant. × 110,000. (c) Glutaraldehyde fixed. × 137,000. (d) Frozen without cryoprotectant. × 83,000.

FIGURE 6 (a–f) These micrographs show the freeze-fractured membrane of the vacuole (CV) adjacent to the adhesion plaque which joins it to the pocket membrane (P). All CVs are highly inflated and apparently preparing themselves for the contraction and discharging events of systole. No connections to spongiome tubules are seen. All frozen without cryoprotectant. (a and b) The E faces of these vacuoles show a characteristic series of deformations (arrowheads) of the CV membrane where it is held against the pocket membrane by the adhesion plaque. Some fibrillar plaque material occupies the space between the two membranes (arrows). × 95,000; × 66,000. (c–f) Contractile vacuoles presumably captured (by ultrarapid freezing) in the process of discharging their contents. Perforations (arrows) in the vacuole membrane and the adhesion plaque could provide channels to the lumen of the pocket. In Fig. 6e, a perforation seems to be bounded by a rim of particles. In Fig. 6f, the pore can be seen to extend through both CV and pocket membranes. × 112,000; × 109,000; × 135,000; × 86,000.
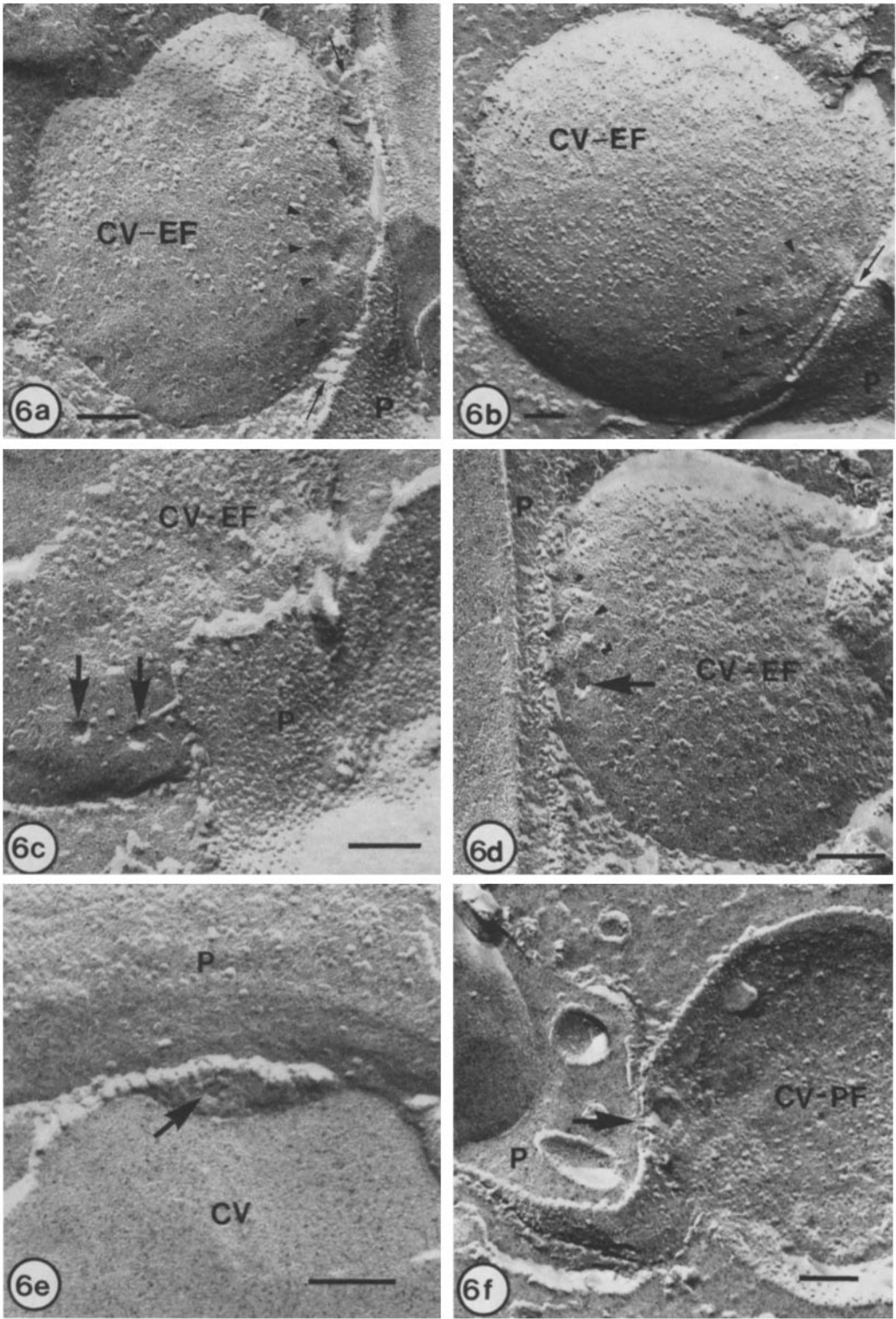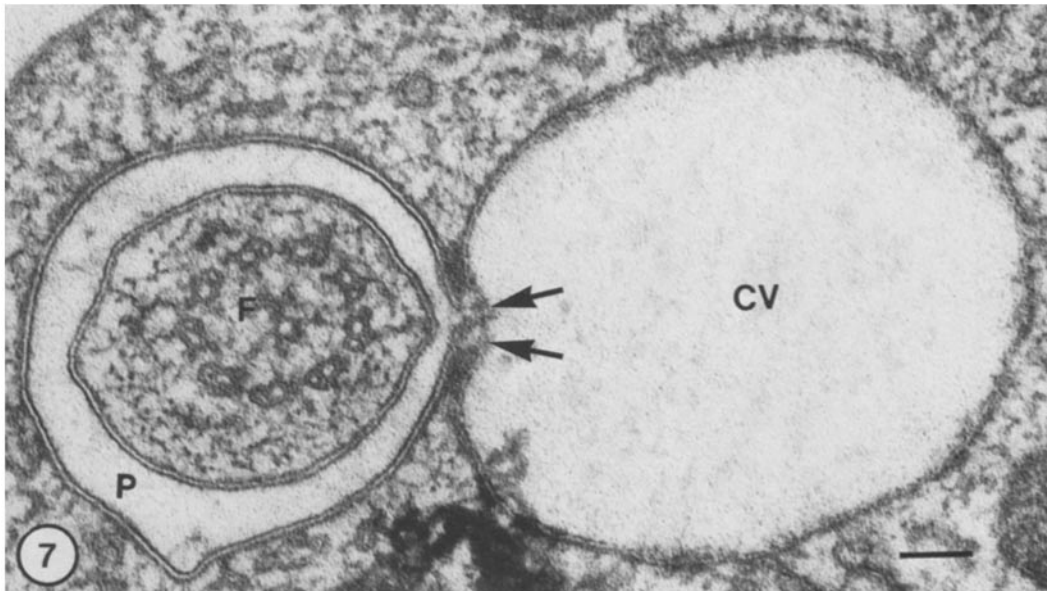
FIGURE 7 Discontinuities are apparent in the dense plaque and associated membranes located between the lumen of the vacuole (*CV*) and the pocket (*P*) (arrows). Note that the two bilayers do not appear to fuse. This vacuole exhibits a patchy coating on its lumenal surface. *F*, flagellum. Freeze-substituted. × 97,000.

ultrarapid freezing methods produce freezing rates 10–30 times faster than conventional methods (6),[1] and few cellular changes should occur during cooling at a rate of 5,000°–10,000° K/s. Additionally, all other membranes appear exceptionally well preserved, and small pores are limited to the adhesion zone of expanded vacuoles with no attached spongiome tubules. Nevertheless, we are not certain whether, even with our rapid freezing techniques, we are preserving all pores in an open configuration or whether some membrane pores close during the freezing event. Other as yet unanswered questions concern the biochemical composition of the plaque material and the trigger necessary to initiate the secretion events.

Because it might be argued that the pores we have observed may not provide a sufficiently large channel for fluid expulsion during the available time interval, we have calculated the pressure required to empty the vacuole by using the Poiseuille equation for the flow of liquid through a tube:

$$P_1 - P_2 = \frac{8 l \eta}{\pi r^4} \frac{dV}{dt}$$

The necessary difference in pressure between the vacuole (P_1) and the outside of the cell (P_2) is a function of l, the length of the pore (measured at 50 nm); r, its radius (15 nm); and η, the viscosity of the fluid (assumed to be that of water, 1.002×10^{-2} g/s-cm). V, the volume of the vacuole, is calculated to be 2.68×10^{-13} cm^3 from its diameter of ~0.8 μm. The duration of systole, t, is assumed to be 0.01 s. For these conditions, we calculate that the pressure required to completely empty the vacuole through just one pore is only 0.67 atm. This small pressure would be further reduced for vacuoles with multiple pores (see Figs. 6c and 7), slower emptying times, and/or vacuoles that are incompletely emptied (Fig. 4a). Thus, this simple calculation indicates that the openings we observe are probably sufficient to permit fluid secretion during the brief period of vacuole contraction.

Diastole

Hypotheses proposed for fluid segregation during diastole include osmosis, phase separation, and active transport (12), but the actual mechanism still remains unknown. McKanna (15, 17, 18) has argued that we must look to the structure of the spongiome tubules for clues about the process of fluid accumulation. He suggests that an important component must be the helically arranged double rows of pegs forming the cytoplasmic coat on all
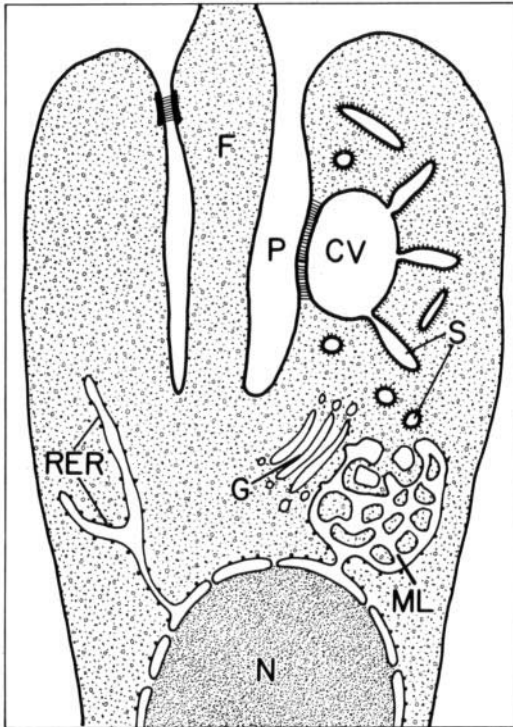
FIGURE 8   Schematic illustration of the organization of the cytoplasm of a *Leptomonas* cell in the vicinity of the contractile vacuole. *N*, nucleus; *RER*, rough endoplasmic reticulum; *ML*, membrane lattice; *G*, Golgi complex; *S*, spongiome tubules; *CV*, vacuole; *P*, pocket; *F*, flagellum.

FIGURE 10   Our reversibly perforated sieve plaque model for fluid secretion. A conformational change in the plaque material may create perforations which leave regions of the lipid bilayer unsupported. Contraction of the vacuole ruptures the bilayers, and fluid is released without membrane fusion. After systole, the conformational change in the plaque is reversed, and the bilayers reseal themselves. *PM*, pocket plasma membrane; *Ad Pl*, adhesion plaque; *CV*, contractile vacuole membrane.
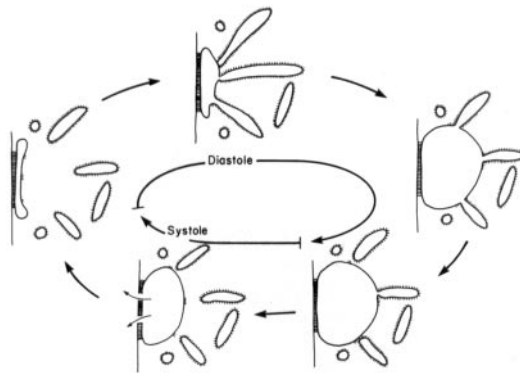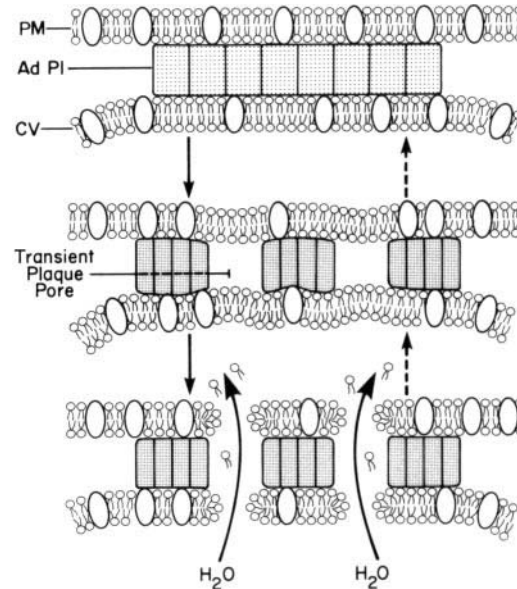

FIGURE 9   This diagram shows our interpretation of the sequence of events during the *Leptomonas* contractile vacuole cycle. During diastole, spongiome tubules fuse to a flattened CV, the vacuole swells, and then the tubules detach. During systole, pores form through the adhesion plaque and both adjacent membranes. Fluid is then released, leaving a flattened vacuole still attached to the side of the pocket.

water-transporting membranes studied. We have now demonstrated an additional specialization of the spongiome tubules: the 11-nm helically arranged intramembrane particles seen on E faces. They may be transmembrane components associated with either the pegs of the cytoplasmic coat or the fuzzy material on the lumenal surface. Other possible functions for these particles include ATP-ase ion pumps or perhaps structural reinforcements to prevent the tubules from ballooning or collapsing under pressure. Alternatively, the particles could be associated with contractile elements that would allow the tubules to undergo contraction-expansion cycles. Peristaltic contractions could then pump fluid toward the vacuole while longitudinal extension and contraction could enable them to make and break the links with the CV.

We propose that the arrays of particles seen on the vacuole are related to the helically arranged, double rows of particles found on the spongiome tubules. They could be left behind on the CV after imprecise fusion and detachment of spongiome

tubules during diastole. Significant numbers of these particle patches could accumulate after several cycles. Because they are found on vacuoles even after ultrarapid freezing, we do not believe that their presence on the vacuoles is an artifact of osmotic swelling or unnatural fusion induced by fixatives or cryoprotectants.

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