GENETIC ANALYSIS OF MEMBRANE DIFFERENTIATION IN *PARAMECIUM*

Freeze-Fracture Study of the Trichocyst Cycle in Wild-Type and Mutant Strains

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ABSTRACT

Using a series of mutants of *Paramecium tetraurelia*, we demonstrate, for the first time, changes in the internal structure of the cell membrane, as revealed by freeze-fracture, that correspond to specific single gene mutations. On the plasma membrane of *Paramecium* circular arrays of particles mark the sites of attachment of the tips of the intracellular secretory organelles—trichocysts. In wild-type paramecia, where attached trichocysts can be expelled by exocytosis under various stimuli, the plasma membrane array is composed of a double outer ring of particles (300 nm in diameter) and inside the ring a central rosette (fusion rosette) of particles (75 nm in diameter). Mutant *nd9*, characterized by a thermosensitive ability to discharge trichocysts, shows the same organization in cells grown at the permissive temperature (18°C), while in cells grown at the nonpermissive temperature (27°C) the rosette is missing. In mutant *tam δ*, characterized by normal but unattached trichocysts, and in mutant *tl*, completely devoid of trichocysts, no rosette is formed and the outer rings always show a modified configuration called “parentheses,” also found in wild-type and in *nd9* (18°C) cells. From this comparison between wild type and mutants, we conclude: (a) that the formation of parentheses is a primary differentiation of the plasma membrane, independent of the presence of trichocysts, while the secondary transformation of parentheses into circular arrays and the formation of the rosette are triggered by interaction between trichocysts and plasma membranes; and (b) that the formation of the rosette is a prerequisite for trichocyst exocytosis.

All freeze-fractured natural membranes studied so far display a smooth background that probably represents regions of lipid bilayer (5, 7), on which “particles” corresponding to proteins or lipo-protein aggregates (9, 20) are usually found. These particles generally appear randomly distributed but various types of organized particle arrays have been described: for instance, the arrays involved in...
FIGURE 1 General organization of *Paramecium*. (a), (b): light micrographs of a silver-stained Paramecium. (a) General organization of the cortex (ventral face). × 600. (b) Detail showing the regular alternation of trichocyst attachment sites (ts) and basal bodies (bb) along the ciliary rows. × 2,000. (c) Low magnification of freeze-etched wild-type plasma membrane, E face (PME). Cilia (ci) and parasomal sacs (ps) are cross-fractured on raised plateaus while the sites of trichocyst attachment alternate in the furrows separating the plateaus. × 10,800.
cell to cell contacts in metazoan cells (17, 23) or the “rosettes” found at the sites of attachment of mucocysts that are involved in exocytosis in the protozoan Tetrahymena (15, 16), or the regular rows of intrathylakoid particles closely connected to the external phycobilisomes in red and blue-green algae (11). The interest of such particle arrays is twofold: (a) with respect to membrane function, by providing a way to correlate local membrane differentiations with particular functions; and (b) with respect to membrane organization, by providing an opportunity to study the genetic and molecular basis of array formation in the fluid membrane matrix.

In Paramecium these two problems can be approached by looking at the arrays found at the sites of attachment of trichocysts. Trichocysts, like Tetrahymena mucocysts, are secretory membrane-bounded vesicles that attach to the plasma membrane at regular intervals over the cell surface. A variety of stimuli induces trichocyst discharge from the cell by exocytosis: the plasma membrane and the membrane surrounding the trichocyst fuse, and the content of the organelle is rearranged into a long needle that is expelled into the medium. The particle arrays that mark the sites of trichocyst attachment on the plasma membrane were first described by Janisch (10), Satir (14), and later, more extensively, by Plattner et al. (12). The arrays consist of a large (300-nm diam) outer ring(s) of ca. 80-Å diam particles and a small (75-nm diam) rosette of ca. 150-Å diam particles in the center of the ring(s). The membrane surrounding the trichocyst displays a densely packed array of particles that forms an annulus of several rows on the tip of the organelle where it makes contact with the plasma membrane.

In an attempt to identify the function of these various particle arrays, especially the ring(s) and the rosettes of the plasma membrane, and to understand the genetic and molecular basis of their formation, we have compared wild-type Paramecium tetraurelia with three mutants: the first completely lacks trichocysts, the second develops normal trichocysts that are unable to attach to the plasma membrane, and the third, a thermosensitive mutant, has trichocysts that attach and discharge normally at 18°C, but when the cells are grown at 27°C, attach but cannot discharge.

Here we are able for the first time to demonstrate changes within the cell membrane that correspond to specific gene mutation. Our results lead to two main conclusions regarding the formation and the function of the plasma membrane arrays: (a) in all three mutants, the outer ring is present, showing that this array develops independently of any interaction with the trichocysts, while its shape and the formation of the rosette are dependent on interactions between trichocyst and plasma membrane; (b) the central rosette is absent in the mutant devoid of trichocysts and in the mutant with no trichocyst attachment, while it is more or less abnormal in the thermosensitive mutant, providing strong support for the hypothesis that the rosette is a prerequisite for exocytosis as suggested previously for the homologous rosettes of Tetrahymena (16).

MATERIALS AND METHODS

Strains and Culture Conditions

Four strains of Paramecium tetraurelia—according to the new nomenclature (22) and formerly P. aurelia, syngen 4—were used: a wild-type strain (stock d4-2), and three mutants: tam 8, a nitrosoguanidine-induced mutant; nd 9, a presumably spontaneous mutant; and tl (trichless), a nitrosoguanidine-induced mutant, obtained and kindly provided by S. Pollack (13). All three mutations are single gene recessive nuclear mutations; tam 8 and nd 9 are genetically independent of each other; allelism of tl with the others has not been tested. The tam 8 mutation causes abnormal macronuclear localization and partition at fission and prevents trichocyst attach-

FIGURES 2 and 3  Organization of trichocyst attachment sites in wild-type cells. × 27,000.

FIGURE 2  Freeze-etched plasma membrane, P face (PMP), showing various aspects of the trichocyst attachment sites. Most are composed of an outer ring of particles and a central “rosette” of particles (r1); where trichocyst extrusion has occurred a crater occupies the rosette’s place (s1).

FIGURE 3  Freeze-etched plasma membrane, E face (PME). Trichocyst sites are recognizable at their expected locations. A few particles and the imprint of the missing ones delineate the outer ring of particles (s1). Particles from the central rosette are visible at some sites (s2). As in fracture face P, discharged trichocysts leave open sites (s3). Notice the lower density of background particles as compared to the density on P face.
ment to the cortex. Trichocysts are formed, but they remain free in the cytoplasm (4). The nd 9 mutation isolated by Brygoo (unpublished observations) prevents trichocyst extrusion, although attachment to the membrane is normal. This is a thermosensitive mutation: no discharge is ever observed when cells are grown at 27°C, while trichocysts are more or less normally discharged by cells grown at 18°C. The nd cells are completely devoid of any structure resembling trichocysts (Table II is provided for reference). Cells were grown in a Scotch grass infusion, bacterized with Klebsiella pneumoniae according to the usual techniques (21) at either 27°C or 18°C. Trichocyst discharge was assayed by treating cells with a saturated picric acid solution and scoring trichocyst expulsion qualitatively under the light microscope (Fig. 12).

Electron Microscopy

Experiments were carried out in two separate laboratories, Gif (G) and Berkeley (B) using slightly different procedures with identical results. Procedure B corresponds to Figs. 4, 5, 15, and 17, procedure (G) to the others.

Conventional Cytology: Cells were either harvested by centrifugation at 2,000 rpm for 7 min at 4°C, fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, and postfixed for 45 min in 1% OsO4 in the same buffer (B) or, alternatively, gently centrifuged at room temperature, fixed with 0.5% glutaraldehyde in 6 mM phosphate buffer, pH 7, for 2 h at room temperature, and postfixed with 1% OsO4 in 25 mM phosphate buffer for 2 h at 4°C (G). Samples were dehydrated in an ethanol series and embedded in Epon or Araldite by conventional methods. Sections were cut on a Reichert (B) or LKB Ultrotome Ill (G) and stained with uranyl acetate and lead citrate. Micrographs were taken on a Siemens 101 electron microscope at 80 kV (B) or Hitachi Hu 12 A at 75 kV (G).

Freeze-etching and Freeze-Fracture Technique: Cultures of Paramecium were harvested as described above. The pellets were incubated in 6 mM phosphate buffer, pH 7, containing 0.5% glutaraldehyde, for 20–30 min at 0°C (G) or at room temperature (G and B).

The slightly fixed cells were washed extensively with phosphate buffer, then transferred to 20% or 30% glycerol for 3 h to overnight. Samples were frozen in liquid Freon 22 (monochlorodifluoromethane), cooled to its freezing point by liquid N2. Freeze-fracture was done by standard techniques, in a Balzers apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) at −100°C or −115°C before casting by Pt-C (G). Replicas were cleaned in sulfochromic acid followed by Eau de Javel or Purex bleach, then extensively washed in distilled water and mounted on 200-mesh copper grids and examined on a Hitachi Hu 12 A electron microscope (G).

RESULTS

Fig. 1 shows the general organization of the cortical structures of P. tetraurelia as revealed by the silver impregnation technique (Fig. 1a and b) and in low-power freeze-fracture replicas (Fig. 1c). Along each ciliary row, kinetosomes and trichocysts alternate regularly. During prefission stages, trichocyst sites can also be found between ciliary rows along secondary meridians. The trichocysts have been studied genetically (13) and biochemically (24), and their development and structure fully analyzed (3, 8). The trichocysts develop in the cytoplasm, then migrate to the cell surface and attach by their tips to the plasma

| Cell type | Fracture face | Number of particles/rosette | Total number of rosettes | Mean number of particles per rosette | Kp |
|-----------|--------------|-----------------------------|--------------------------|------------------------------------|----|
| Wt 27°C   | P            | 0 1 1 1 6 7 21 8 4 4        | 52                       | 8                                  | 5  |
|           | E            | 19 16 20 13 3 1 1           | 73                       | 1.6                                |    |
| Wt 18°C   | P            | 6 1 2 1 6 7 12 13 6 1       | 55                       | 6                                  | 2.9|
|           | E            | 15 16 24 18 11 3            | 89                       | 2.1                                |    |
| nd9 27°C  | P            | 73 20 10 6 3 1              | 113                      | 0.6                                | *  |
|           | E            | 35 15 6 6 3                | 65                       | 0.8                                |    |
| nd9 18°C  | P            | 7 1 7 8 8 11 10 6 5 1       | 65                       | 4.4                                | 2.9|
|           | E            | 15 10 8 5 6                | 44                       | 1.4                                |    |

The figures represent, for the P and E faces obtained from wild-type and mutant nd9 cells grown at 27°C and 18°C, the number of rosettes composed of 0, 1, 2, 3 and more particles. Partition coefficients (Kp) are given in the last column, except in the case of nd9 at 27°C (*). Where too few particles are involved.

Wt, wild-type.
membrane sites where they rest until discharge is triggered. Each trichocyst is bounded by a unit membrane. Some trichocysts discharge normally as the animal swims, but massive discharge can be induced experimentally, for example by picric acid, our routine assay for trichocyst discharge ability (Fig. 12). When a trichocyst discharges, its membrane fuses with the plasma membrane and the content is reorganized into a periodic, needle-like structure. The membrane differentiations involved in normal trichocyst attachment and discharge have been examined previously in wild-type Paramecium; here we compare wild-type to mutant cells defective in one step of the trichocyst cycle (development, attachment, or discharge).

Dифференциации в Wild-Type Cells

Плазматическая мембрана: как и в других мембранах, замораживание и дробление плазматической мембраны Paramecium показывает две дополнительные стороны; Fig. 4 и 5 Тонкие срезы через расположенные и вытягивающиеся трихоцисты.

Рисунок 4 Трехоцистовский кончик вставлен между двумя подпелликуллярными альвеолами (spa), а апикальная часть трехоцистовой мембраны образует небольшую выпучину (стрелка) похожую на маленькую клюк, показанную на рис. 8. Трехоцистовая мембрана (tm); трехоцистовский кончик (t). × 120,000.

Рисунок 5 Срез перпендикулярный к поверхности клетки показывает трехоцист в процессе вытягивания: его мембрана (tm) сливается с плазматической мембраной (PM), релиз трехоциста. × 38,000.

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one, the P or protoplasmic fracture face (6), is densely particulate; the other, the E or exterior fracture face, contains fewer particles. This difference is illustrated in Figs. 2 and 3. The arrays of particles corresponding to the trichocyst attachment sites have a particle partition coefficient ($K_p$) greater than one (19), and are therefore best seen on fracture face P (Fig. 2).

In agreement with previous observations, three distinct zones are conveniently distinguished within resting trichocyst sites: (a) a single or double ring of particles (10, 12) ca. 300 nm in diameter comprising an average number of $2 \times 40$ particles whose size does not exceed 110 Å. This defines the outer limit of the trichocyst attachment site; (b) a central rosette (12, 14) 75 nm in diameter composed of an average of eight particles (Table I) whose size reaches about 150 Å with a $K_p$ of 5 at 27°C; and (c) a zone of exclusion, between the outer ring and the rosette, containing disperse and heterogeneous particles.

While this description corresponds to the most frequently seen trichocyst sites, two main variations are found. First, as previously observed by Janisch (10), instead of being circular, the outer ring may look more or less collapsed, as in Fig. 15. We will refer to this state as “parenthesis.” Here no central rosette is observed. Second, the center of the outer ring is sometimes occupied by a “hole” of variable size, which may be considered as the visualization of steps in the process of trichocyst extrusion. This hole appears as a depression on fracture face P (Fig. 2) and as a crater on fracture face E (Fig. 3). This is quite comparable to the pictures of mucocyst extrusion described by Satir (16) in Tetrahymena: because of this similarity, the process of exocytosis and membrane fusion, illustrated in Figs. 5, 8, 9, therefore does not need further description. During the discharge process, the outer ring (which is absent in Tetrahymena) seems to get wider as the hole enlarges. It is worth mentioning that images of trichocyst discharges are never observed when cells are fixed at 4°C (G).

For all the observations just described, no differences in gross appearance between plasma membranes of cells grown at 27°C or 18°C could be found. However, for central rosette particles, for which we have more quantitative data, the mean number of particles per rosette increases somewhat on the P fracture face and in total, and the $K_p$ rises (Table I) when the cells are grown at 27°C.

**TRICHOYSTS AND THEIR RELATION TO THE PLASMA MEMBRANE:** The relationships between the plasma membrane and the trichocyst (a) in resting position and (b) during extrusion are illustrated in thin sections in Figs. 4 and 5, respectively. Fig. 4 shows that the elongated trichocyst tip comes to lie in close apposition to the plasma membrane, in between adjacent subpellicular alveoli. At the center of the tip is an apical protrusion or knob (Figs. 4 and 8), in the region that we think underlies the rosette of the plasma membrane, the radius of curvature of the trichocyst membrane changes abruptly and the contact between the two membranes becomes much more intimate. In Fig. 5 the trichocyst is extruded into the medium, and the trichocyst membrane has become continuous with the plasma membrane.

The trichocyst membrane (Figs. 7–8) is ornamented by a complex array of tightly packed particles: at least three annuli differing in size and arrangement of particles can be distinguished. The $K_p$s of these arrays are much greater than 1.

The rosette of the plasma membrane overlies the

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**FIGURES 6–9** Relationship of plasma membrane and trichocysts.

**FIGURE 6** A favourable fracture through plasma membrane, P face (PMP), and cytoplasm (cy) showing on the right the outer ring of particles (or) above the underlying positioned trichocyst and, on the left, the central rosette (r) and, right beneath, a trichocyst tip (fracture face E of the trichocyst membrane) inserted between subpellicular alveoli (spa). × 54,000.

**FIGURE 7** Annulus of particles on trichocyst membrane (a). P face. × 72,000.

**FIGURE 8** Two intracellular aspects of trichocysts: the tip of one, still undischarged, shows a little apical knob matching the central rosette of the plasma membrane; the other is in the process of extrusion and is swollen. × 33,800.

**FIGURE 9** Stages of trichocyst discharge equivalent to that of Fig. 5. Notice the presence of particles (arrow) at the junction between plasma membrane (PM) and trichocyst membrane (tm). × 40,500.
Figure 10. Organization of trichocyst sites in mutant nd9 grown at the permissive temperature (18°C), P face (PMP). (a) Outer rings (or) and central rosettes (r) comparable to those of wild-type are present. × 20,250. (b–d) Stages in the cycle of trichocyst sites. (b) Circular outer ring without rosette. (c) Circular outer ring with a rosette. (d) Early stage in discharge. × 40,500.
central portion of the trichocyst (Fig. 8), where the knob is apparently located. Below the complex annular arrays are impressions of striations that are probably indentations caused by the microtubular bundle that surrounds the organelle (3). Without etching, these appear only on the P face (Figs. 8 and 20 e). During discharge, the trichocyst membrane greatly swells and rounds up into a truncated sphere (Fig. 9). The striations disappear in the rounded membranes and few particles are seen on either the P or E fracture face. Although our images clearly demonstrate continuity between plasma and trichocyst membranes around the open lip, we are unable to decide as yet the fate of the annular arrays.

**Differentiations in Mutant Cells**

**Plasma Membrane:** For the thermosensitive mutant *nd9*, the trichocysts are attached to the plasma membrane regardless of growth temperature (18°C or 27°C), but they can be discharged only by cells grown at 18°C. An aspect of plasma membrane fracture face P of *nd9* cells grown at 18°C is illustrated in Fig. 10. The trichocyst sites are comparable to those found in wild-type: typical outer ring, central rosette, and discharging sites are seen. The only differences that we have been able to measure so far concern the number of particles in the central rosette, which is lower than in wild-type cells grown at 27°C, but might not be different from the number in wild-type cells grown at 18°C where a wide spread of measurements is also found (Fig. 11 and Table I). If the number of particles were lower in mutant than in wild-type, this could be correlated with the fact that at 18°C, *nd9* cells, although they can discharge their trichocysts under stimulation by picric acid, do so less massively than the wild-type cells.

Fractures of *nd9* cells grown at 27°C are quite different (Figs. 12–14). On the P face (Fig. 13), the outer ring is always circular and quite comparable in diameter and in numbers of particles to that of wild-type membranes, but it is generally devoid of a central rosette, or, if a rosette is present at all, there is a very reduced number of particles at the rosette site (Fig. 17, Table I). On the corresponding E face (Fig. 14), one sees the trichocyst site imprint with few particles from the outer ring and essentially none from the central rosette.

As for the mutants with either no trichocysts attached (*tam 8*) or no trichocysts at all (*th*), the P faces have two striking features in common illustrated in Fig. 15: (a) the outer ring is present but almost always displays the “parenthesis” configuration sometimes observed in wild-type membranes, and (b) no central rosette is ever found. As far as we have determined, other properties of the fracture face, including the relative positions of cilia and trichocysts and general appearance of the background particles and so on, remain the same as those of wild-type in all these mutants.

**Trichocysts and Their Relation to the Plasma Membrane:** Comparison of *nd9* cells grown at 18°C or 27°C to wild-type reveals no detectable differences in the fracture face.
faces of the trichocysts themselves. Fig. 18 shows P and E faces of the trichocyst tip in the mutant at 27°C, which displays the same arrays of particles in the same position as described for the wild-type (Figs. 7, 8). Furthermore, in nd9 cells, even under nonpermissive conditions, the positioning of the trichocysts and their relations to the plasma membrane and subpellicular alveoli are normal. In contrast, in tam 8 cells, where the trichocysts are not found attached to the plasma membrane but only in the cytoplasm, these trichocysts are generally devoid of annuli (Fig. 23 c) even though their overall structure in thin section is normal. In one instance, a trichocyst in the cytoplasm has been observed to possess a loosely organized annulus, which is not at its normal apical location (Fig. 19 b). In tl and tam 8 cells, at positions where trichocysts are normally encountered in wild-type there is a gap in the alveolar system that is filled with an electron-dense amorphous mass in place of a mature trichocyst tip (Fig. 17). Cross-fractures of the hillock region are devoid of trichocyst membrane fracture faces. tl cytoplasm contains numerous membrane-bounded vesicles but no obvious trichocyst precursors (Fig. 16).

DISCUSSION

The trichocyst cycle in P. aurelia comprises at least three major steps: (a) morphogenesis in the cytoplasm; (b) migration and attachment of the mature organelle to the plasma membrane; and (c) exocytosis. The latter two steps involve interactions with the plasma membrane which can be visualized by the appearance of particle arrays on the trichocyst membrane and on the plasma membrane, and subsequent changes in these arrays. The trichocyst cycle can be dissected effectively and the functional significance of the arrays analyzed by the study of a series of mutants blocked at different steps in the developmental sequence. We have examined three such mutants: tl, blocked at the morphogenetic level; tam 8, blocked at either migration or attachment; and nd9, a temperature-sensitive mutant, blocked at exocytosis at the nonpermissive temperature (Table II).

The plasma membrane arrays, which we have examined in greater detail, consist of a fusion rosette and surrounding rings. We have also described a particular configuration of the ring which we call a parenthesis; the particles of rings and parentheses are the same size and number and partition similarly. The parenthesis is found occasionally in wild-type, in nd9 cells grown at 18°C, and consistently in tam 8 and tl mutants. Our results regarding the occurrence of these arrays in the various mutants are summarized in Table II and Figs. 20–23.

The first general conclusion that can be drawn from the results is that there are rings or parentheses devoid of rosettes, but that every rosette is surrounded by rings. This indicates that these two sets of particles assemble independently, and that the parentheses and, probably, the rings develop before the rosettes appear.

Since parentheses are always present at all the potential trichocyst attachment sites in cells which have no trichocysts at all (tl) or no trichocysts attached (tam 8), it appears that the parenthesis develops independently of any interaction with the trichocyst membrane. Furthermore, since trichocysts can be expelled by nd9 cells grown at 18°C as they are from wild-type cells, and since some trichocysts are “spontaneously” expelled by living cells at any time, it is quite reasonable to assume that in these two cell types, in which a minority of parentheses are found, some sites are not occupied by trichocysts. On the contrary, in nd9 cells grown at 27°C, in which trichocysts are attached but never expelled and where nearly 100% of the rings are circular, it is reasonable to assume that all or almost all the sites are occupied by trichocysts. Therefore we conclude that (a) the

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**Figures 12–14** Organization of trichocyst sites in mutant nd9 grown at the nonpermissive temperature (27°C).

**Figure 12** Light micrograph of picric acid treatment on wild-type (WT) and mutant nd9 (m). No trichocysts are expelled by the mutant. × 150.

**Figure 13** P face. Trichocyst sites are normally aligned along ciliary rows and some (ts*) between the rows (a feature of predivision stage). The outer double ring of particles is the same as in wild-type, but central rosettes are never observed. Cross-fractured cilium (ci). × 27,000.

**Figure 14** E face. A few particles from the outer ring are visible, but none in the location of the central rosette. × 27,000.
FIGURE 15 Fracture P face of mutant tam 8. The outer ring of particles always presents the collapsed aspect of "parentheses," also visible at some sites in wild-type membranes. All sites are devoid of trichocysts, and no central rosette is found. × 27,000.

FIGURE 16 Cross-fracture of mutant tl showing one unoccupied trichocyst site. × 60,000.

FIGURE 17 Thin section of mutant tl showing a similar area. Note the dense material where a trichocyst tip would be normally located. × 120,250.
Figure 18 Fracture through the pellicle of mutant nd9 (27°C) showing a cilium with its ciliary necklace (cn) and patches (p) and two trichocyst tips. On the right, a fracture face P (tm1) with an annulus; on the left (tm2), the imprint of the same array on the E face of the trichocyst membrane. × 40,500.

Parenthesis represents a normal precursor form of the ring and corresponds to unfilled trichocyst attachment sites, whereas the ring corresponds to filled attachment sites. (b) the parentheses arrays assemble in the plasma membrane independently of any interaction with trichocyst, and (c) trichocyst attachment probably occurs before formation of the rosette.

Plattner et al. (12) interpret the ring particles as the points of connection between the plasma membrane and the membrane of the subpellicular alveoli. Our results support this conjecture. We find gaps in the alveolar system at trichocyst attachment sites regardless of whether trichocysts are actually present or not. When trichocysts are present, however, the gap increases as if the alveoli were pushed apart to make room for the trichocyst tip (Fig. 4). This probably corresponds to the conversion of a parenthesis into a true ring. Thus, although the initial formation of this array is independent of the trichocyst, the final form depends on this organelle. The intramembrane particles of the parenthesis are pushed apart through the fluid background of membrane lipids because they are attached to the retreating alveoli.

The function of the parentheses or rings is unclear. It must be pointed out that in *Tetrahymena*, alveoli are also interrupted around cilia and mucocyst sites, but the rosettes are not surrounded by rings. This, together with the observation that normal-appearing rings are present in *Paramecium* unable to discharge trichocysts, suggests that the ring is not an essential feature for exocytosis itself.

However, during exocytosis, the diameter of the fusion pocket initially grows to fill the ring, so that the ring may eventually prevent further expansion of the pocket and delimit the fusion event. The fate of the ring in the later stages of exocytosis is still uncertain in our studies. In any case, it remains to be understood why and how the parentheses originally develop on each membrane ridge between cilia territories, i.e., how the cell patterning is programmed at the plasma membrane level.

Our results yield information on the function of the rosette. In support of the hypothesis of Satir et al. (16, 18) regarding the homologous rosette of *Tetrahymena*, and in contrast to the ring, the
rosette appears to be an essential feature of the process of exocytosis of Paramecium trichocysts. The rosettes are present only when the trichocysts are attached (wild-type and nd9 at permissive temperature), but trichocyst attachment alone is not sufficient for rosette formation. In the absence of a normal rosette, in nd9 cells at 27°C, there is no trichocyst discharge, while the same mutant recovers, at the permissive temperature, both its rosette particles and its discharge capacity. To our knowledge, this is the first direct demonstration, based on both genetic and structural evidence, of the precise function of a membrane particle array.

Regarding the rosette particles, the data in Table I suggest that discharge may be quantitatively related to the total number of rosette particles in that either (a) discharge may be triggered only when some critical number of rosette particles is present, or (b) the probability of discharge rises as the number of rosette particles increases. In any case, a minimum of five particles would be required for exocytosis, since no discharge was observed in nd9 cells grown at 27°C where practically no rosette with more than four particles was found (Table I).

It is also interesting that despite the difference in total particle numbers, the Kps of wild-type and mutant rosettes at 18°C are virtually identical. Kp is apparently a measure of relative bonding strength to either side of a freeze-fracture particle,
FIGURES 20–23  Summary of the main features of wild-type and mutant strains.

FIGURE 20  Wild-type arrays; (a, c) P face; (b, d) E face. x 81,000; (e) x 80,000; and (f) x 81,600: annulus on trichocyst membranes on complementary P and E faces.

FIGURE 21  Mutant nd9 grown at 18°C. (a, b) Aspect of rosette and outer ring on P and E faces. x 81,000.

FIGURE 22  Mutant nd9 grown at 27°C. (a, b) Aspect of outer ring without central rosette on P and E faces. x 81,000.

FIGURE 23  Mutant tam 8. (a, b) Outer ring in "parenthesis" on P and E faces, x 81,000; (c) intracellular trichocyst, E face, without annulus, x 54,000.
which varies over a wide range for different arrays, but is relatively constant for a single class of particles (19). We conclude that the rosette particles in wild-type and mutant at 18°C are possibly identical in bonding strength to either side of the membrane—that is, their insertion into the surrounding membrane is similar. This is not surprising in view of the fact that the physiological function of the rosette is retained. On the other hand, at 27°C, the partition coefficient changes for wild-type. Aside from being the first so far reported variation of \( K_p \) as a function of temperature, this may be indicative of changes in the intrinsic environment of the particle and hence its ability to function during exocytosis. This remains to be studied further.

With respect to the mechanism of fusion, Ahkong et al. (1) have postulated that it may involve an increase in lipid fluidity in the region of fusion where freeze-fracture particles will be excluded. Allison and Davies (2) suggest that increase of lipid fluidity is a function of the radius of curvature of the membrane: the smaller the radius of curvature, the greater the possibility of fusion. The knob that we have described at the trichocyst tip may therefore be a specific differentiation of the trichocyst membrane that promotes fusion (Figs. 4 and 8). This knob lies directly below the interior of the fusion rosette, which conforms to the model of the Lucy group (1).

We have not yet studied the formation of the annular arrays on the trichocyst membrane in as great detail as the rosette. An apparently normal annulus is always present on attached trichocysts, not only in wild-type and \( nd^9 \) at 18°C, but also in the undischargeable trichocysts of \( nd^9 \) at 27°C. Therefore, the formation of the annulus per se is not sufficient for exocytosis. Although the annulus is usually absent on \( tam^8 \) unattached trichocyst tips, even when these are fractured through what seems to be their full length, in one instance a trichocyst in the cytoplasm was observed to possess a loosely organized annulus that was not in its usual apical location (Fig. 19 b). It seems that the appearance of the annulus and its migration to a fixed apical location are late stages in trichocyst maturation that normally postdate attachment. However, annular array formation can seemingly rarely occur in the cytoplasm without attachment. It has not been possible to find unattached mature trichocysts in the wild-type cytoplasm.

In *Tetrahymena*, upon membrane fusion the annulus everts and apparently acts as a restraining ring, to prevent unscheduled rupture of the membrane, and as a barrier in the plane of the membrane that segregates components during incorporation (16). These features could be possibly the same for *Paramecium*.

A last point deserves some comment. If our conclusion that "parentheses" correspond to unoccupied sites and rings to occupied ones is valid, this may mean that trichocyst attachment brings about a spatial rearrangement of the ring particles in the plane of the membrane and that these particles migrate within the lipid bilayer. An alternative possibility would be that the conversion of parenthesis to ring occurs via stretching of the plasma membrane around the trichocyst tip. A more striking example of membrane fluidity might be provided by the observation, previously mentioned, that during prefission stages rings or parentheses are found also between the longitudinal rows of kinetosomes. It is tempting to assume that these trichocyst sites are first formed between ciliary rows, and later migrate to their final position along these rows, after membrane elongation at fission has provided room for them between duplicated kinetosomes.

This discussion has indicated the range of information on membrane organization, differentiation, and function that can be derived from correlated genetic, physiological, and structural studies on a series of mutant cells. The stringency of genetic control of membrane processes is particularly evident. More information about the nature of the localized structure and function of the fluid mosaic membrane is likely to be obtained as various additional mutants are brought under examination in further studies now underway.

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