Control of Exocytotic Processes: Cytological and Physiological Studies of Trichocyst Mutants in *Paramecium tetraurelia*

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ABSTRACT The trichocysts of *Paramecium tetraurelia* consitute a favorable system for studying secretory processes because of the numerous available mutations that block, at various stages, the development of these secretory vesicles, their migration towards and interaction with the cell surface, and their exocytosis.

Previous studies of several mutants provided information (*a*) on the assembly and function of the intramembranous particles arrays in the plasma membrane at trichocyst attachment sites, (*b*) on the autonomous motility of trichocysts, required for attachment to the cortex, and (*c*) on a diffusible cytoplasmic factor whose interaction with both trichocyst and plasma membrane is required for exocytosis to take place.

We describe here the properties of four more mutants deficient in exocytosis ability, *nd6*, *nd7*, *tam38*, and *tam6*, which were analyzed by freeze-fracture, microinjection of trichocysts, and assay for repair of the mutational defect through cell-cell interaction during conjugation with wild-type cells. As well as providing confirmation of previous conclusions, our observations show that the mutations *nd6* and *tam6* (which display striking abnormalities in their plasma membrane particle arrays and are reparable through cell-cell contact but not by microinjection of cytoplasm) affect two distinct properties of the plasma membrane, whereas the other two mutations affect different properties of the trichocysts. Altogether, the mutants so far analyzed now provide a rather comprehensive view of the steps and functions involved in secretory processes in *Paramecium* and demonstrate that two steps of these processes, trichocyst attachment to the plasma membrane and exocytosis, depend upon specific properties of both the secretory vesicle and the plasma membrane.

The trichocysts of *Paramecium* are secretory vesicles whose formation in the cytoplasm, migration to the cell cortex, interaction with the plasma membrane, and exocytosis are easily observable by light and electron microscopy and are also amenable to genetic dissection. A number of mutations are available that block trichocyst development and exocytosis at various stages. These mutations disclose different steps and functions that might not be suspected or identified in other systems. The trichocyst system offers two further advantages for studying secretory processes.

First, in the region of contact between trichocyst and plasma membrane, organized arrays of intramembrane particles are visible on freeze-fracture replicas of both the plasma and the trichocyst membranes (1, 7, 16, 22, 27). The presence and/or configuration of these particle arrays are altered by mutations and can, therefore, be correlated with a precise function in the exocytotic process.

Second, two types of biological assays can be used to obtain information on the site and mode of action of the mutations; by microinjection of trichocysts, it is possible to localize the mutational defect in either the plasma membrane, the trichocyst, or the cytoplasm, as first demonstrated by Aufderheide (2), and by observation of mutant cells paired to wild-type partners during conjugation, one can assess whether the effect of the mutation can be repaired by cell-cell contact (6, 9, 15).

Previous studies of several mutants have already led to some

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understanding of the assembly and function of intramembrane particles arrays (7), to the demonstration of an autonomous motility of trichocysts that can be abolished by mutation (2, 3), to the precise localization in the "rosette" (an intramembrane particle array of the plasma membrane at the site of trichocyst attachment) of a Ca^{++} ATPase activity (23, 24), and to the identification of a readily diffusible cytoplasmic product whose interaction with the plasma membrane and the trichocyst membrane is necessary for exocytosis (6, 14).

In this paper, we analyze the effects of four more mutations (*tam6*, *tam38*, *nd6*, and *nd7*) by freeze-fracture analysis, microinjection experiments, and observation of repair of the mutant phenotypes during conjugation. Our findings show that two of the four mutations affect plasma membrane function and organization, whereas the other two affect trichocyst properties. We provide additional evidence for the dependence of exocytotic capacity upon the presence of complete rosettes and we confirm that the "annulus" at the trichocyst tip is assembled upon trichocyst interaction with the cell surface. These new data, added to those previously obtained by various studies of other mutants, provide a rather comprehensive view of the functions involved in the secretory process in *Paramecium* as based on the study of a total of 10 mutants. The bearing of these data on the understanding of the control of exocytosis processes in general is discussed.

MATERIALS AND METHODS

Strains and Culture Conditions

The strains of *Paramecium tetraurelia* used in these experiments or cited for comparison are listed in Table I. All the mutants correspond to recessive, monogenic mutations, each belonging to a different complementation group (11). The cells were grown according to established procedures (28) in Scotch grass infusion inoculated with *Klebsiella pneumoniae* (nonpathogenic strain) the day before use. Culture temperature was 27°C unless otherwise specified.

Freeze-Fracture Analysis and Microinjection Experiments

Freeze-fracture analysis was carried out as previously described (6).

Microinjections, serving to transfer cytoplasm and uninserted trichocysts from cells of one genetic type to another, were performed as has been previously described (2, 18, 19). 2 h after injection, the host cells were tested for exocytotic competence by killing them with a 1% aqueous solution of tannic acid or a mixture of 1:3 of saturated picric acid and 1% tannic acid. The discharge of even a single trichocyst can be detected by observation under dark-field, low-power optics (3, 25).

TABLE I

List of the Strains Used or Cited

Strain	Phenotypic properties	Reference*
WT	Normal trichocysts, attached, excretable	
tam6	Normal trichocysts, unattached	8
nd6	Normal trichocysts, attached, unexcretable	This paper
tam38	Abortive trichocysts, football shaped, unattached	26
nd7	Normal trichocysts, attached, unexcretable	This paper
nd9	Normal trichocysts, attached, unexcretable at 28°C, excretable at 18°C	7
tam8	Normal trichocysts, unattached	8
ftA	Abortive trichocysts, unattached	25
tl	Lacking recognizable trichocysts	25
ndA	Normal trichocysts, unattached	25
ndB	Normal trichocysts, attached, nonexcretable	25

* The reference cited corresponds to the first published study on the different mutants. Information on all strains is also given in Sonneborn (28).

The established protocol for trichocyst microinjection (2) tests independently the functional competence of the trichocysts and of the nontrichocyst cytoplasm, which is apparently essential for development of in vivo exocytotic potential. Fig. 1 illustrates the principle of the method.

In a first step, a reference recipient strain m_0 is defined. A sample of m_0 cells, which carry abortive trichocysts unable to be excreted, receive a sample of wild-type (WT) cytoplasm containing a few trichocysts. These WT trichocysts can attach to the plasma membrane of the m_0 recipient cell and can be excreted upon stimulation by the standard picric or tannic acid tests. It is then concluded that the m_0 recipient cell lacks functional trichocysts and possesses all other necessary cytoplasmic and plasma membrane functions. Usually, cells homozygous for the *ftA* mutation are used for m_0. The functional competence of mutant trichocysts is then tested by placing them into *ftA* cells. After an appropriate postinjection wait, injected *ftA* cells are tested with picric or tannic acid. If discharge is seen, it is concluded that the injected trichocysts are capable of normal function in a genetically complementary cytoplasm and are, therefore, competent. If discharge is not seen, it is concluded that the mutant trichocysts retain their mutant phenotype even in cytoplasm where WT trichocysts function normally. They are, therefore, considered to be defective. Conversely, competence of the cytoplasms of mutant cells is tested by injecting WT trichocysts into the mutants and scoring the resulting exocytotic response. Three results are possible: *a*, no discharge, which indicates that the mutant cytoplasm is unable to use normal trichocysts and is, therefore, defective; *b*, discharge of a few trichocysts from a few cells, which indicates that the mutant cytoplasm is competent because it can use normal trichocysts when these are provided; or *c*, discharge of hundreds to thousands of trichocysts from virtually all injected cells, which indicates that the cytoplasm of the mutant being tested is defective but has been "repaired" by some nontrichocyst cytoplasmic factor transferred by the injection. This last conclusion is further tested by injecting *ftA* cytoplasm into the mutant cells. If valid, the same repairlike

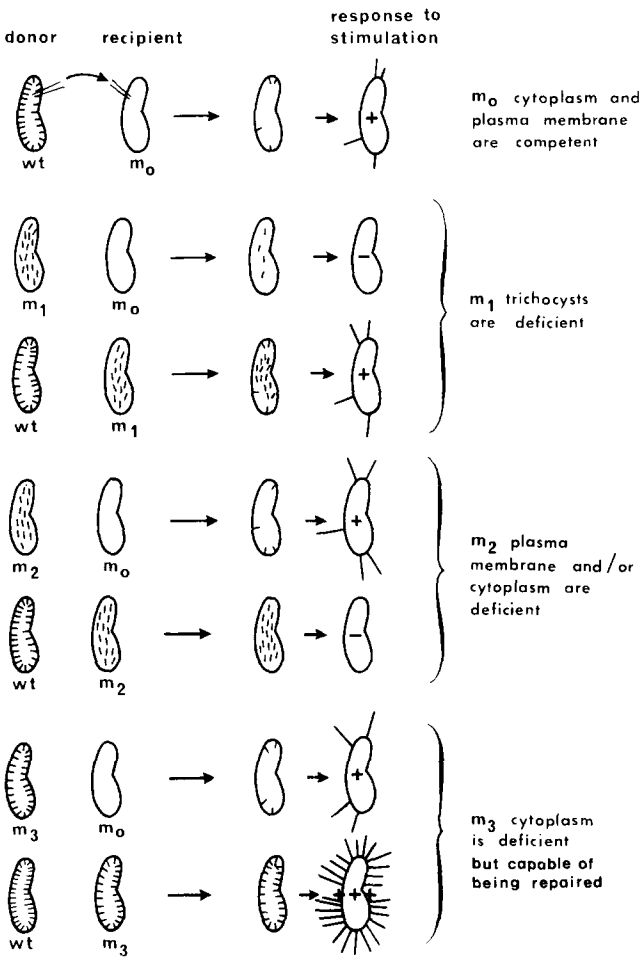


FIGURE 1 Schematic diagram outlining the procedures and conclusions to be drawn from the various microinjection tests of trichocyst function. For a detailed explanation, see Materials and Methods.

result as with WT should occur. Thus, one can score a particular mutant type as having defective or normal trichocysts and defective or normal cytoplasm, on the basis of interpretation of the microinjection tests.

Conjugation Experiments

During conjugation, paired paramecia remain united for 5.5–6 h at 27°C. The exchange of gametic nuclei, which will provide both partners with an identical heterozygous nuclear complement, occurs by the 4th hour. It is known that electrical coupling between conjugants is achieved by the 2nd hour (Y. Naitoh, personal communication) and the labeled amino acids diffuse from one conjugant to the other (9). Under normal conditions (i.e., in the absence of cytoplasmic bridges which may develop at the end of the conjugation period [28, 29]), mitochondria are not exchanged, as demonstrated in crosses between cells carrying different mitochondrial markers (21). It is reasonable to assume that trichocysts, whose size is bigger than that of mitochondria, are not exchanged either. For trichocysts or behavioral mutations, whose phenotype can be ascertained by a rapid test, it is possible to observe the restoration of a WT phenotype to the mutant by its WT conjugant partner. In three previously reported cases, restoration of a WT phenotype was observed as soon as 2–2.5 h after pairing, i.e., before nuclear exchange, which occurs by the 4th hour of pairing. The *pwA* (9) or *cnrC* (15) conjugants, paired with a WT partner, acquire normal capacity for ciliary reversal and the *nd9* (27°C) conjugants recover normal trichocyst exocytotic capacity in *nd9* × wt pairs (6). Because the restoration occurs before nuclear exchange, it can be concluded that repair of the mutational defect is caused either by diffusion of WT gene products into the mutant partner or by some membrane change induced in the mutant by cellular contact with WT.

The conjugation method was used in the following way. Sexually reactive mutant and WT cells were mixed and a pool of tightly united pairs was isolated after 1.5 h. Such pairs are well synchronized in their nuclear processes (28) and will all separate by 5.5–6 h after mixing and agglutination. At chosen times, a sample of pairs was tested by addition of picric acid and examined under the microscope.

RESULTS

The trichocysts of *Paramecium* are complex, paracrystalline membrane-bound structures (5-μm long) which develop in the cytoplasm. They then migrate to the cell surface and attach at preformed sites of the cortex, where they can remain until their excretion is triggered by external stimuli which all seem to induce a Ca^{++} influx (24). Unstimulated WT paramecia have a few thousand trichocysts associated with their cell surfaces (25). Cortically attached trichocysts are located along the ciliary rows, at the anterior and posterior boundaries of each ciliary unit. Upon stimulation, exocytosis is triggered and is easily monitored under light microscopy as the discharging trichocyst bodies transform into 20-μm long paracrystalline "needles", forming a fringe around the cell.

More than 25 mutants affecting the trichocyst cycle have been isolated and genetically analyzed (11, 25, 29). Their common property (which generally was used to screen the mutants) is to be defective in exocytotic capacity; upon stimulation, no (or very few) trichocysts are expelled. All these mutants fall into three categories: *a*, mutants that form normal trichocysts normally attached to the cortex but unable to be excreted; *b*, mutants that form normal trichocysts unable to attach; and *c*, mutants with no trichocysts or with structurally abnormal ones. The first category of mutants allows one to identify steps and functions in trichocyst exocytosis, whereas the last two categories allow one to analyze the developmental pathway of the organelle.

The properties of four mutants were analyzed and are reported here. The mutants *nd6* and *nd7* are representative of category *a*, the mutant *tam6* belongs to category *b*, and the mutant *tam38* to category *c*.

Freeze-Fracture Study

As previously described (1, 7, 16, 22, 27), three types of intramembrane particle arrays, illustrated in Fig. 2, are correlated with interaction between plasma membrane and trichocyst. First, in the plasma membrane, an array consisting of ~80 particles arranged in the form of a "parenthesis" preexists at each presumptive site of trichocyst attachment (Fig. 2 *a*). Upon trichocyst attachment, the parenthesis is transformed into a double ring ~30 nm in diameter and a central rosette of 8–10 larger particles (15 nm) is assembled (Fig. 2 *b*). Second, at the tip of attached trichocysts, at the point of closest contact with the plasma membrane, an annulus of several tight rows of intramembrane particles is observed (Fig. 2 *c*). Previous data (1, 7) suggested that this annulus is assembled only at the time of trichocyst attachment. The presence and/or the organization of these intramembrane particle arrays were examined in the mutants *nd6*, *tam6*, *nd7*, and *tam38*.

In the mutant *nd6*, trichocysts are attached but unable to be excreted. Its main features revealed by freeze-fracture are a normal aspect of trichocysts and some defects in the plasma membrane arrays. On P fracture faces of the plasma membrane (Fig. 3 *a*), at trichocyst sites, two types of abnormalities are observed; at some sites the ring has a somewhat irregular configuration (Fig. 3 *b*), whereas at other sites it seems well organized but unusually large. 44 rings on P fracture faces of *nd6* cells were measured (in millimeters) on electron micrographs × 27,750. In Fig. 5, the distribution of *nd6* ring diameters is compared with that of 44 WT rings and of 44 rings of another mutant, *nd7*. The mean diameter of *nd6* rings is significantly larger than that of WT cells (see legend of Fig. 5) and corresponds to an actual mean size of 0.33 μm, as compared with 0.29 μm for WT. Most of the sites are devoid of any normal rosettes (Fig. 3 *b*); as shown in Fig. 6 *A*, eight out of 40 examined sites display only one to three rosette particles. No rosette particles are present on E fracture faces. Attached trichocysts (Fig. 4 *a* and *b*) display, at their apical end, a normal annulus, whereas this particle array is absent on unattached trichocysts free in the cytoplasm.

The mutant *tam6*, which prevents trichocysts attachment, is leaky and has a thermosensitive expression. At its restrictive

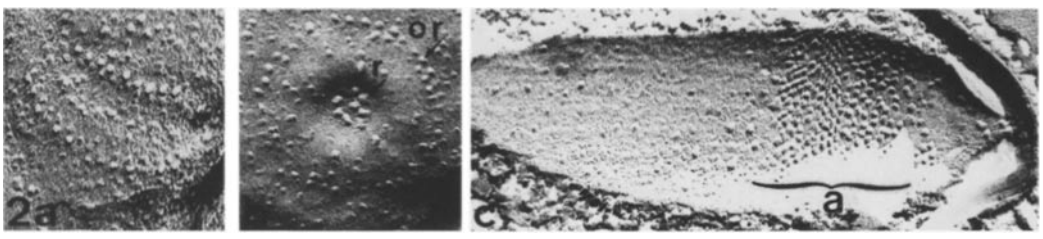
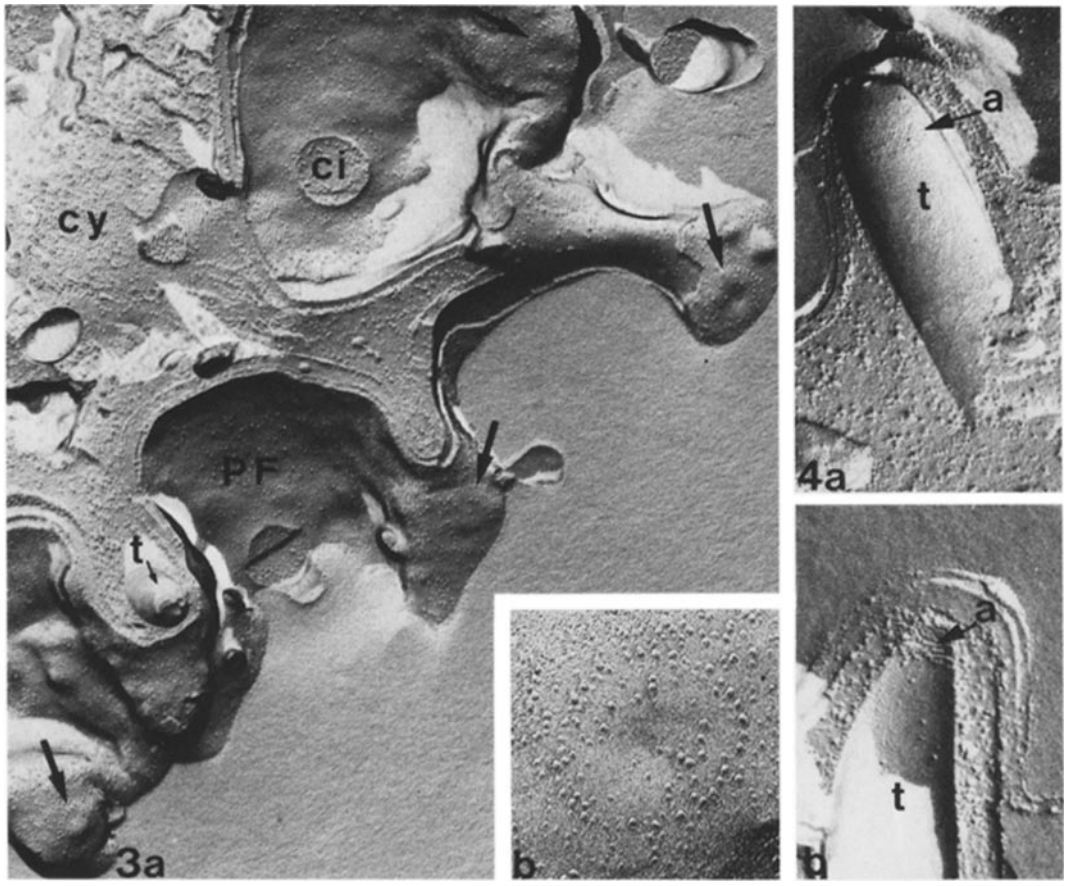


FIGURE 2 Intramembrane particle arrays involved in trichocyst-plasma membrane interactions. (a) P fracture faces of "parenthesis" (× 81,000). (*b*) P fracture faces of "outer ring" (*or*) and its central rosette (× 81,000). (c) P fracture face of "annulus" (a) at the trichocyst tip (× 91,000).



FIGURES 3 and 4 The mutant *nd6*. (3 *a*) Aspect of cytoplasm (*ct*) and of P fracture face (*PF*) of plasma membrane with three trichocyst attachment sites (arrows). *ci*, Cilium. × 27,700. (3 *b*) Enlarged site showing an abnormal-looking outer ring without a central rosette × 78,600. (4 *a*) E fracture face of a positioned trichocyst (*t*) with the apparent imprint of the annulus (*a*). × 41,600. (4 *b*) P fracture face of a positioned trichocyst (*t*) with its annulus (*a*). × 55,600.

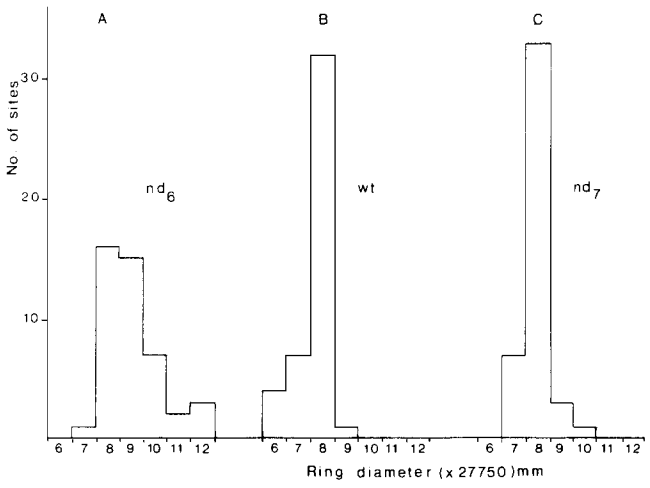


FIGURE 5 Diameter of the outer rings in the mutant *nd6*, WT cells, and the mutant *nd7*. In abcissa, the scale in millimeters corresponds to the measurements carried out on pictures like Fig. 3, at the magnification × 27,750, and the classes correspond to 1-mm differences. In ordinate, the number of rings in each class. 44 sites were measured on *nd6* P fracture faces and the same number of sites for WT and *nd7*. The means and their standard error are, respectively, 9.25 ± 0.19 mm for *nd6*, 8.13 ± 0.09 mm for WT, and 8.04 ± 0.11 for *nd7*.

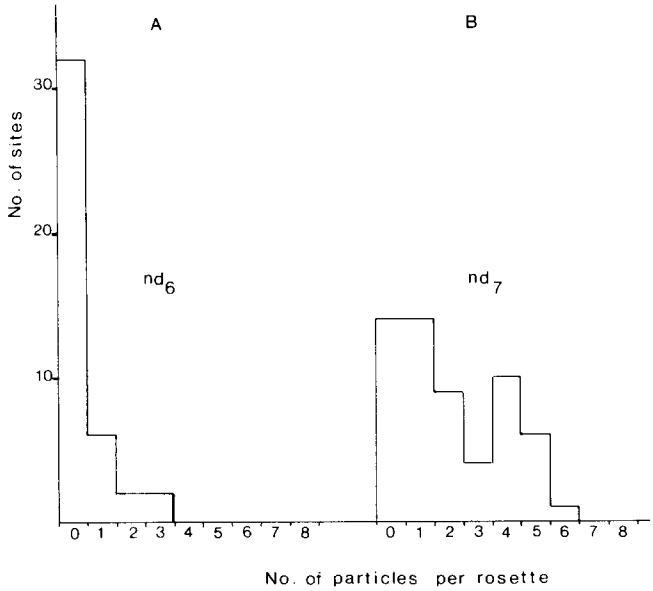
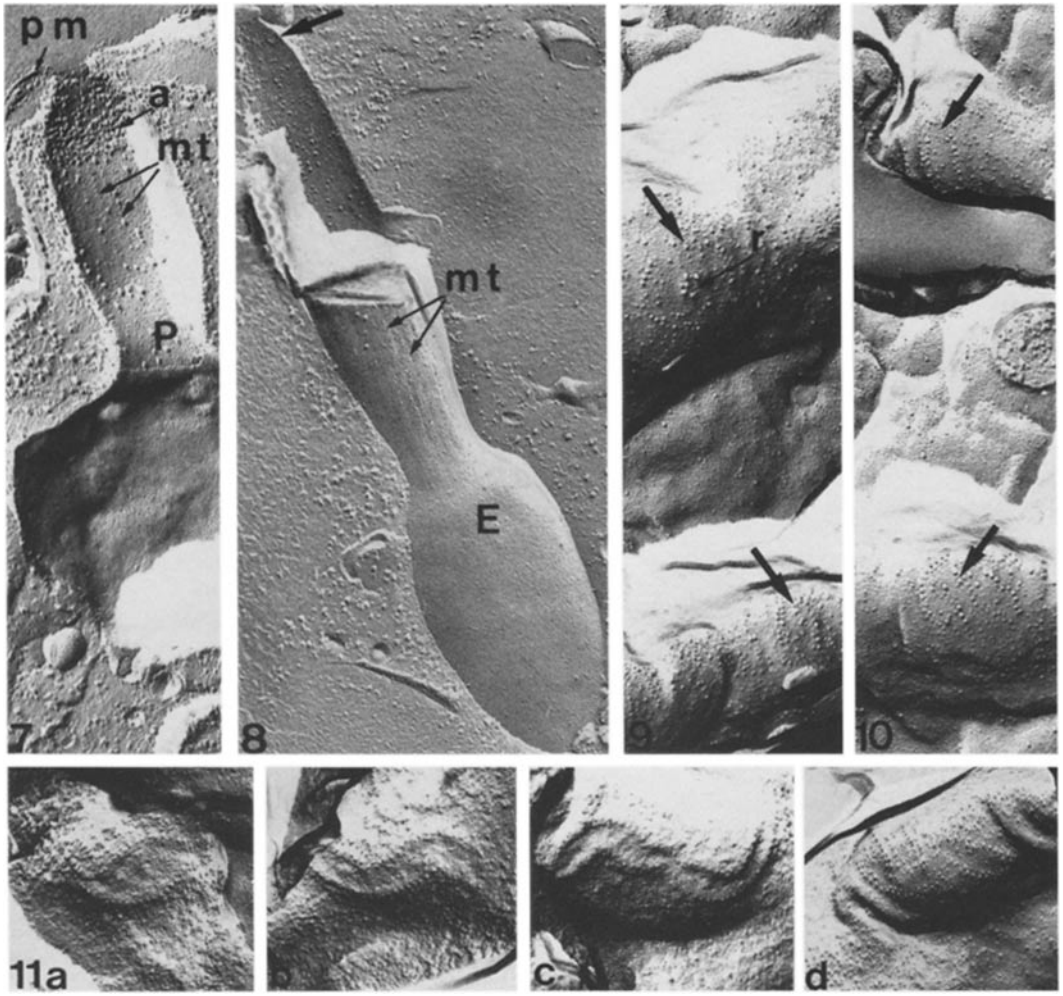


FIGURE 6 Number of rosette particles per site in the mutants *nd6* and *nd7*. Rosette particles are defined by their size and their grouping in the center of the ring. 58 sites were examined for *nd7* and 40 for *nd6*. The mean number of rosette particles per site is eight in WT cells (7).

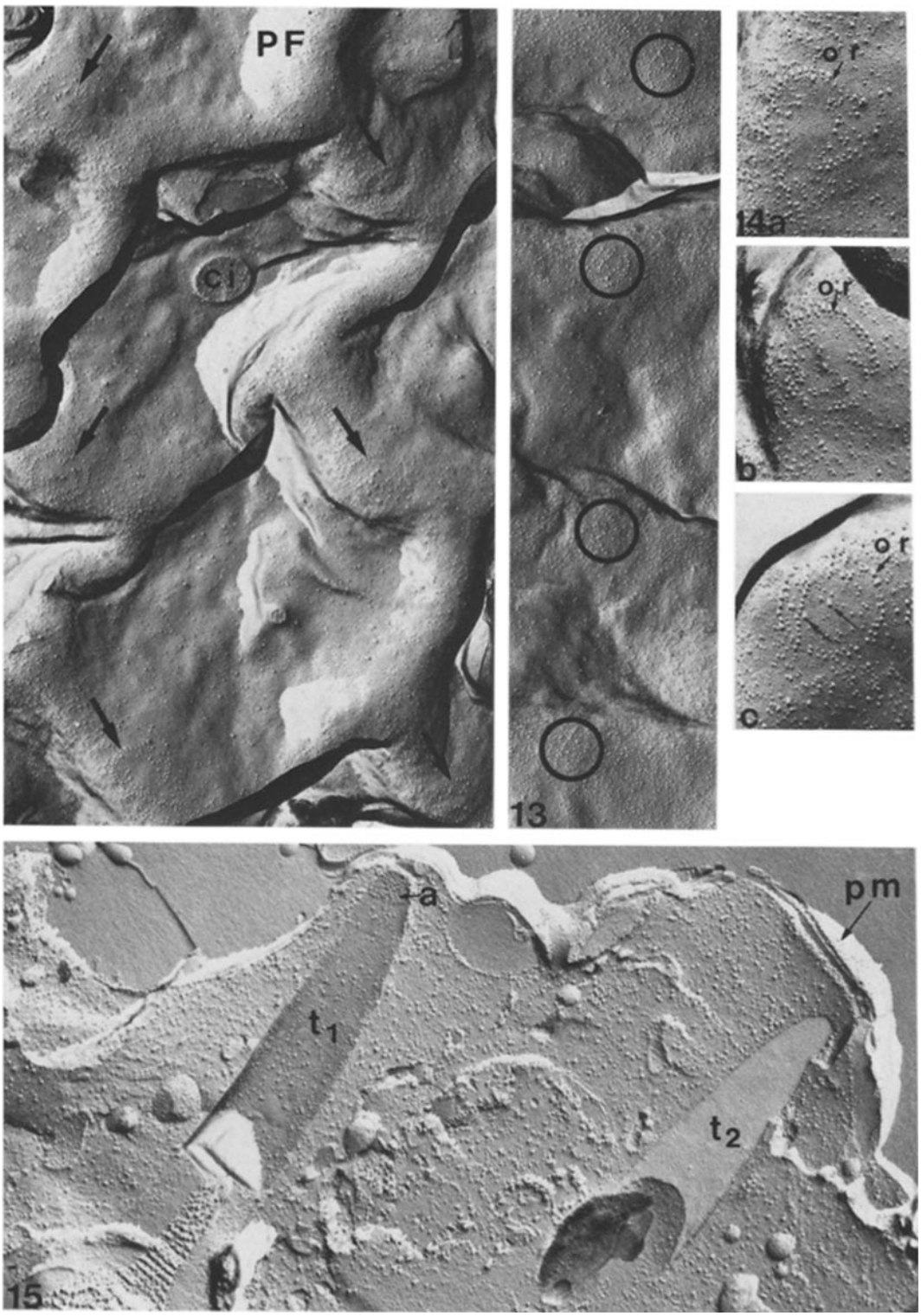
temperatures (18°C or below), most trichocysts are free in the cytoplasm and the cortex is virtually unoccupied. At 28°C, a significant number of trichocysts can attach and be excreted, although the cytoplasm is still loaded with unattached trichocysts. Only cells grown at 18° and 13°C were examined. As in the case of *nd6*, the main features of this mutant as seen in freeze-fracturing are abnormal configuration of plasma membrane arrays and normal trichocysts. One attached trichocyst (Fig 7) shows a normal annulus and the imprints of the microtubule shaft first described by Bannister (5). It can be pointed out that particles seem lined up along the microtubule imprints. On unattached trichocysts, as expected, the annulus is absent, whereas the imprint of microtubule shaft is visible (Fig. 8). In the plasma membrane, a few normal sites with typical rosettes (Fig. 9) are seen, these most likely to correspond to the minority of attached trichocysts. Most sites, however, display unusual or disorganized configurations (Fig. 10), stretched parentheses (Fig. 11 *a*), or a striking type of twisted double parenthesis (Fig. 11 *b–d*).

The mutant *nd7* is characterized by attached trichocysts incapable of excretion. Freeze-fracture reveals normal or subnormal rings with frequent incomplete rosettes. In the P fracture face of the plasma membrane, as shown in Fig. 12, rings can be observed, whereas in Fig. 13, parentheses smaller than those in WT are seen. The mean diameter of the ring (Fig. 5 *c*) is identical to that of WT cells. The ring often appears to be composed of two "independent" half circles, a characteristic which had not been previously reported but which is also present in some WT fracture faces. In some cases, one half-circle appears regular, the other half dispersed (Fig. 14 *a–c*). Large particles similar to rosette particles are observed inside the ring but in unusual locations (Fig. 14 *a* and *c*). In 50% of the sites, two to six rosette particles were counted (Fig. 6 *b*). It is of particular interest to note that *nd7* cells can occasionally excrete one or few trichocysts and that the Ca^{++} ATPase activity, which had been shown to be located in the rosette and is absent in all mutants devoid of rosettes, can be only inconsistently demonstrated in *nd7* cells (24). When attached trichocysts are fractured, an annulus of particles, possibly incomplete, is observed (Fig. 15).

In the mutant *tam38*, abortive "football"-shaped trichocysts similar to those first described by Pollack (25) show the char-



FIGURES 7–11 The mutant *tam6*. Fig. 7 shows the P fracture face (*P*) of a positioned trichocyst. The annulus (*a*) is present and particles may have migrated from the base to the end of the trichocyst tip along the imprints of microtubules (*mt*). *pm*, Plasma membrane. × 54,000. Fig. 8 shows the E fracture face (*E*) of a cytoplasmic trichocyst. The morphology is normal. The annulus is not yet organized; the arrow indicates the presumptive site of its assembly. Microtubule imprints (*mt*) are visible. × 40,500. Figs. 9 and 10 show three types of sites. In Fig. 9, the upper site is subnormal with a nearly complete rosette. In Fig. 10, the lower site is disorganized. The other two sites correspond to normal parentheses. × 36,000. Fig. 11 shows examples of abnormal plasma membrane arrays. *a*, Stretched parenthesis; *b–d*, elongated or twisted double parentheses. × 40,500.



FIGURES 12–15 The mutant *nd7*. Fig. 12 shows the plasma membrane P fracture face (*PF*) showing three occupied trichocyst sites. *ci*, Cilium. × 27,700. Fig. 13 shows the plasma membrane P fracture face with four aligned "small" parentheses (circle). × 27,700. Figs. 14 *a*–*c* show enlargements of plasma membrane sites. Outer ring (*or*) of particles where one-half is normal and the other half dispersed. Dispersed large rosette like particles are visible inside the rings (arrows). × 50,900. Fig. 15 shows the fracture through the cortex showing two trichocyst tips (t_1), P fracture face with annulus (*a*); (t_2), E fracture face. *pm*, Plasma membrane. × 34,800.

acteristic elongated tip with the annulus. However, *tam38* trichocysts display on the P fracture faces (Fig. 16) irregular rows of particles whose imprint is visible on E faces. In the plasma membrane (Figs. 17 and 18), only parentheses are observed, in agreement with previous data (7) showing that this configuration corresponds to the preformed plasma membrane attachment site before trichocyst attachment. However, many of these parentheses appear longer than normal (0.37 μm). They often appear as "double" parentheses (Fig. 18). It could not be ascertained whether this double configuration

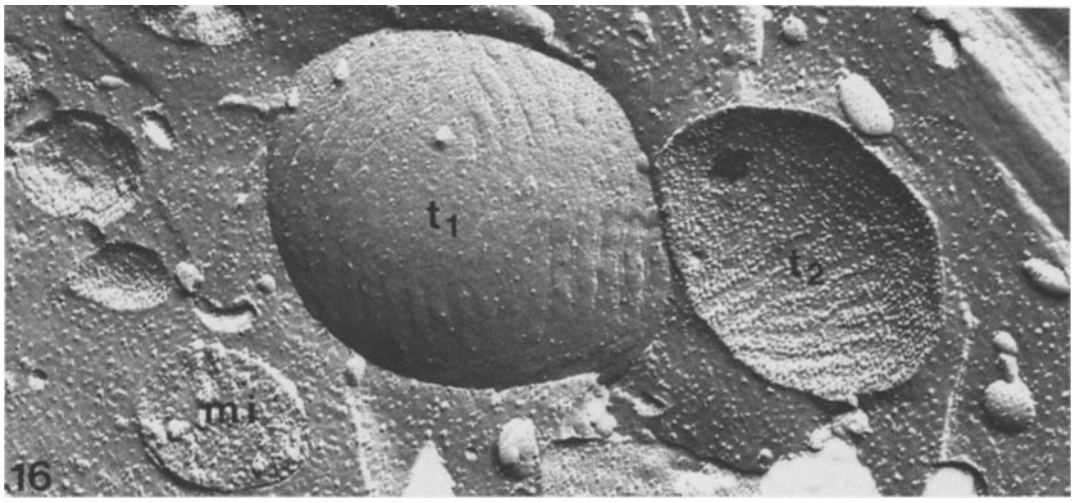


FIGURE 16 Trichocysts of mutant *tam38* with football phenotype. (t_1), E fracture face; (t_2), P fracture face. The ridges with numerous particles on the P face correspond to the grooves on the E face. *mi*, Mitochondrion. × 41,700.

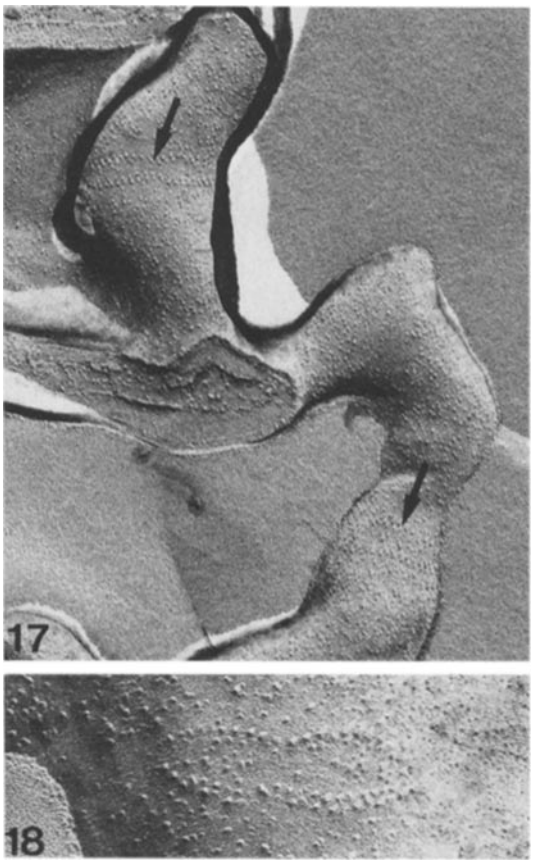


FIGURE 17 Plasma membrane P fracture face of the mutant *tam38* with two unoccupied trichocyst sites (arrows). × 40,500.

FIGURE 18 Enlargement of a trichocyst site in double parenthesis. × 81,000.

corresponded to actually abnormal configurations or to replication forms.

Microinjection Experiments

As first shown by Aufderheide (2) and explained in the Materials and Methods, it is possible to assess which cellular "compartment" (trichocyst, plasma membrane, and/or cytoplasm) is primarily affected by a mutation using a microinjection protocol. The properties of the trichocysts from the four mutants were tested by injection into *ftA* recipient cells and conversely, the properties of their cytoplasm and plasma membrane tested by injection of WT trichocysts. The results are given in Table II. In addition to the results concerning the four mutants, results obtained by Aufderheide (2) in the analysis of mutants *ftA* and *nd9* are also indicated for comparison. In *nd7* and *tam38* cells, WT trichocysts can attach and be excreted. The primary effect of these mutations bears on the properties of trichocysts. In contrast, in *nd6* and *tam6* cells, WT trichocysts supplied by microinjection cannot be excreted. However, *nd6* or *tam6* trichocysts can attach and be excreted when in a competent recipient cell (*ftA*). Therefore, the primary defect of the *nd6* and *tam6* mutations is either in the plasma membrane and/or in the cytoplasm. Furthermore, neither WT into *nd6* nor WT into *tam6* yield a massive and general positive response similar to the response observed for *nd9* cells injected with WT cytoplasm. This fact indicates that, in contrast to the mutation *nd9* (2, 6, 14), the *tam6* and *nd6* mutant phenotypes are not repaired by a WT cytoplasmic component, or at least that such a factor is not present in sufficient quantity in the injected sample of cytoplasm (~5,000 μm^3) to effect a repair.

Repair of Mutation Defects by Cellular Contact During Conjugation

As described in Materials and Methods, the repair of mutational defect by WT diffusible cytoplasmic factors can also be detected by observing the trichocyst phenotype of the paired cells during conjugation. This method differs from the microinjection technique in two ways; the nature and amount of the factors received by the mutant cell may be different and membrane contacts between paired mutant and WT cells may influence the mutant phenotype.

In a first set of experiments, the phenotype evolution of mutant WT pairs was followed and two types of results were repeatedly obtained. (*a*) For two mutants, *nd7* and *tam38*, the mutant conjugants remained incapable of excreting trichocysts until sometime after separation of the conjugants. (*b*) For the other two mutants, *nd6* and *tam6*, a WT or sub-WT phenotype was observed in the mutant conjugant within 4 h of pairing.

However, the accuracy of these observations, particularly in the case of unreparable mutants, might have been hampered by the massive trichocyst discharge of the WT conjugant; a limited repair in the mutant conjugant might have been overlooked. To clear up the picture, further observations were carried out on pairs in which both partners, carrying different and complementary mutations, were initially defective in exocytotic capacity. A number of crosses between each mutant and various other mutants genetically complementary to it were followed. The observations concerning *nd6*, *nd7*, and *tam38* are reported in Table III. For comparison, the results of four crosses involving the mutant *nd9*, whose phenotype is known to be reparable by microinjection of $nd9^+$ cytoplasm (2, 6), are also shown. It can be seen that *tam38* and *nd7* conjugants retain their mutant phenotype until the end of conjugation, whereas *nd6* conjugants acquire exocytotic capacity, as *nd9* conjugants do, before the 3rd hour of pairing. Fig. 19 shows the type of responses obtained. As visible on Fig. 19 *a–c*, representing *nd6* × *nd7* pairs at 1.5, 2, and 3 h, in nd6 conjugants, repair always begins at the zones of contact between the paired cells and then progressively extends to the rest of the cortex. This mode of repair is different from what is observed in the case of an *nd9* cell; in this case, exocytotic capacity is restored simultaneously over the whole cell surface and no intermediate stage is observable between those illustrated in Fig. 19 *a* and *d*. This difference between *nd6* and *nd9* conjugants was repeatedly observed and must be considered as quite significant.

For the mutant *tam6*, which is leaky and can always excrete some trichocysts, significant results could not be obtained by the picric acid test of exocytotic capacity. The effect of conjugation was, therefore, studied by in vivo observation. Because the cytoplasm of *tam6* cells is loaded with unattached trichocysts, the presence of many attached trichocysts was taken as the criterion for restoration of a WT phenotype. A quite significant and progressive repair was observed between the third and fourth h of pairing.

TABLE II

Localization of the Site of Action of the Mutations by Microinjection

Donor	Recipient	No. of cells showing positive response	Total no. of cells observed
tam6	*ftA*	8	18
ftA	*tam6*	0*	18
ndA	*ftA*	7	18
WT	*nd6*	1†	18
	nd6 (uninjected)	3	50
nd7	*ftA*	0	19
WT	*nd7*	7	16
	nd7 (uninjected)	7	92
WT	*tam38*	4	20
WT§	*ftA*	28	113
WT‖	*nd9* 27°C	19**	20

The positive response of injected cells corresponds to the excretion of one to many trichocysts upon stimulation by picric acid, as described in Materials and Methods.

* The *tam6* mutation is leaky and most cells can excrete a variable number of trichocysts (generally much lower than WT cells). The figure 0* simply indicates that none of the injected cell excreted more trichocysts than uninjected control *tam6* cells.

† The *nd6* mutation shows a very low leakiness; out of 53 uninjected control *nd6* cells, three excreted one to three trichocysts. The single positive response from injected cells is not considered significant.

§ Running total of Aufderheide's data, including previously published results (2).

‖ From Aufderheide (2).

** In this experiment, the positive response of *nd9* recipient cells is not only general but massive. This fact has been shown to result from the repair of the mutational defect by a cytoplasmic factor present in WT cells (2, 6, 14).

TABLE III

Repair of the Mutant Phenotype during Conjugation

Cross	Time of observation (h) 2.5	3.5	4.5	5.5	6.5
nd9 × *nd6*	0/10	8/12	7/8		
nd7 × *nd6*	0/7	8/12	8/8		
tam6 × *nd6*	0/10	4/10	10/11		
tam8 × *nd6*		2/8	9/9		
nd6 × *nd7*	0/7	0/12	0/8		2/10
nd9 × *nd7*	0/15	0/13	0/9		
tam6 × *nd7*	0/10	0/11	0/12		
nd9 × *tam38*				0/7	2/7
nd6 × *tam38*				0/7	6/11
nd6 × *nd9*	0/10	12/12	8/8		
nd7 × *nd9*	1/15	13/13			
tam8 × *nd9*	8/8		9/9		

Each mutant was crossed to several nonallelic mutants. Time 0 corresponds to the mixing of sexually reactive cells. Tight pairs were isolated after 1.5 h and a sample of pairs was examined by the picric test at different times during conjugation or just after separation (6.5 h). The figures represent the number of positive responses to the picric acid test over the total number of examined pairs. The indicated response of each mutant was inferred from the known responses of the partner mutant, all results being cross-consistent. For example, *tam8* or *nd7* remaining nonexocytotic throughout conjugation, appearance of an exocytotic response in one conjugant permitted us to ascertain its genotype.

From the comparison of microinjection and conjugation results it appears that the two techniques provide indeed complementary information; whereas the effects of the mutations *tam38* and *nd7* are not reparable by either technique, the effects of the other two mutations, *tam6* and *nd6*, which are not reparable by microinjection of WT cytoplasm, are reparable by physical contact during conjugation with a $tam6^+$ or $nd6^+$ partner, respectively.

DISCUSSION

In *P. tetraurelia*, the control of secretory processes can be analyzed by using mutations that block trichocyst development and exocytosis at various stages.

The mutations can be characterized in two ways. (*a*) Cytologically, it is possible to detect the effects of the mutations on the presence and/or organization of the membranar differentiations involved in trichocysts-plasma membrane interactions, e.g, in the plasma membrane, parentheses, outer rings, and rosettes and, in the trichocyst membrane, annulus at the tip of the organelle. (*b*) Physiologically, it is possible to localize the site of action of a mutation (in the trichocyst itself, the cytoplasm, or the plasma membrane) by microinjection (2) or conjugation (7) experiments.

Previous studies have shown that trichocyst attachment to the plasma membrane can be blocked by mutations affecting only the trichocyst compartment and that exocytosis of attached trichocysts can be blocked by mutations affecting either the trichocyst compartment (2) or a cytoplasmic diffusible produce interacting with both trichocyst and plasma membrane (6).

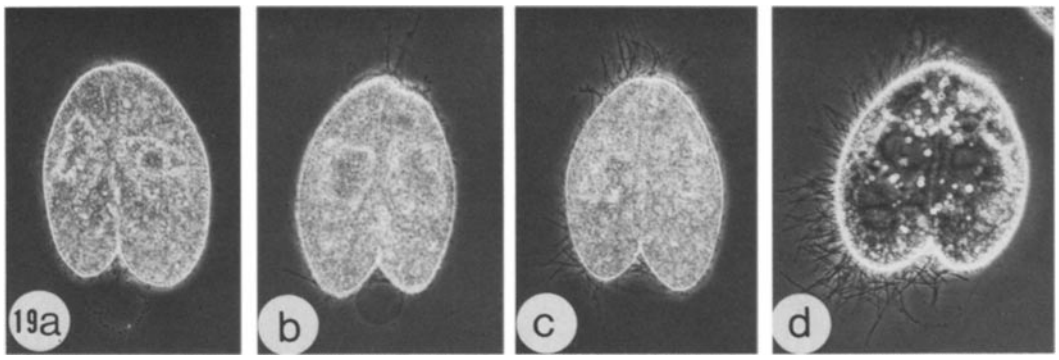


FIGURE 19 Repair of the mutant phenotype during conjugation. *a–c*, Three pairs of the same cross *nd7* × *nd6* fixed with picric acid 1.5 h after mixing of the two sexually reactive cells (*a*), 2 h after mixing (*b*), and 3 h after mixing (*c*). Trichocysts are excreted by the *nd6* conjugant mostly in the region of contact between the conjugants. (*d*) A typical image of an *nd7* × *nd9* pair of conjugants at 2.5 h, showing massive trichocyst excretion over the entire surface of the *nd9* conjugant. The nd7 partner remains "bald" because it is not repaired during conjugation.

Further analysis of the control of secretory processes was carried out by studying four new mutants *nd6*, *nd7*, *tam6*, and *tam38*. The properties of these mutants fully confirm previous conclusions concerning the function and/or assembly of intramembrane particle arrays and demonstrate the existence of a new class of mutations whose site of action lies in the plasma membrane.

The idea that exocytotic capacity is dependent upon the presence of a normal rosette (6, 7) is confirmed here by the mutant *nd6*, which has attached trichocysts but no rosettes, the mutant *nd7* which shows, at best, incomplete rosettes (Fig. 12–15), and also by the leaky mutant *tam6* in which the few normal rosettes probably correspond to the few sites where trichocysts are attached and excreted. The conclusion that parentheses are assembled in the plasma membrane independent of any interaction with trichocysts (7) is confirmed here by the mutants *tam38* and *tam6*, which carry only or mostly unoccupied sites. Although we do not know whether the long or double parentheses observed in *tam38* cells represent abnormal configurations or duplicating sites, all sites in this mutant unambiguously belong to the parenthesis class, and in *tam6* cells the best organized sites also clearly belong to the parenthesis class (Fig. 11). Finally, it had been suggested (1, 7) that assembly of the annulus at the apex of trichocyst membrane was triggered by trichocyst attachment to the cortex. This is confirmed here. In *tam6*, *nd6*, and *nd7* cells, both attached and unattached trichocysts were freeze-fractured; only the attached ones displayed the annulus. The correlation is particularly significant in the case of the mutants *nd6* and *tam6*, which are shown by microinjection experiments to carry functionally normal trichocysts that can be attached and excreted by competent recipient cells.

The mutants *tam6* and *nd6* define a new class of mutations. Cytologically, these two mutants are the only ones presenting gross abnormalities in the configuration of their plasma membrane particle arrays. Physiologically, these two mutants differ from those whose defect lies in the trichocyst (and cannot be repaired by either microinjection or conjugation) and from the mutant *nd9* (2, 6), which is defective in a cytoplasmic diffusible factor and can be repaired both by microinjection of WT cytoplasm and by cellular contact with an *nd9*$^+$ partner. The mutants *nd6* and *tam6* carry functional trichocysts, and their defect is only reparable through cellular contact with a WT (or a complementary mutant) partner. Furthermore, restoration of exocytotic capacity in *nd6* conjugants proceeds in a polarized way, starting in the region of contact with the nd6$^+$ conjugant, as illustrated in Fig. 19. This phenomenon, which is not observed for the mutant *nd9* (Fig. 19*d*), strongly suggests that the repairing factors progress along the cortex rather than by diffusion throughout the cell (as in the case of *nd9*). Altogether, the cytological and physiological observations support the conclusion that the mutational defect in *tam6* and *nd6* affect some properties of the plasma membrane that control the organization of its particle arrays and its functional interaction with the trichocysts.

The results presented here by confirming and extending the information derived from the study of other mutants lead to a fairly precise description of the trichocyst secretory pathway and its genetic control. This pathway, pictured in Fig. 20, is based upon the data shown in Table IV which summarizes the properties of the 10 mutants now studied, including the four described here.

The morphogenesis of trichocysts involves two independent pathways: (*a*) the morphogenesis of the secretory vesicle per se, which can be blocked at different stages by numerous mutations yielding abnormal or incomplete structures and (*b*) the acquisition by the trichocyst of an autonomous motility, required for its migration towards and attachment to the cell surface (3). The independence of the two pathways is demonstrated by the fact that all nonmotile trichocysts studied are apparently structurally normal, whereas all the abortive trichocysts are motile.

In the plasma membrane, parentheses differentiate at each presumptive site of trichocysts attachment before any interaction with the organelle and, therefore, seem to depend upon specific properties of the plasma membrane. One mutation, *tam6*, shown here to affect plasma membrane properties, is also found to alter parentheses organization.

The functional interactions between plasma membrane and trichocysts operate at two levels; attachment of trichocysts, which triggers circularization of the parenthesis and assembly of the rosettes (seat of a Ca^{++} ATPase activity) (23) and exocytosis in response to external stimuli. Attachment of trichocysts can be prevented by mutations altering either the trichocyst (*tam38*) or the plasma membrane (*tam6*). Circularization of the parenthesis seems to correspond to a mere physical response to trichocyst attachment; particles of the parentheses rearrange in the plane of the plasma membrane and no muta-

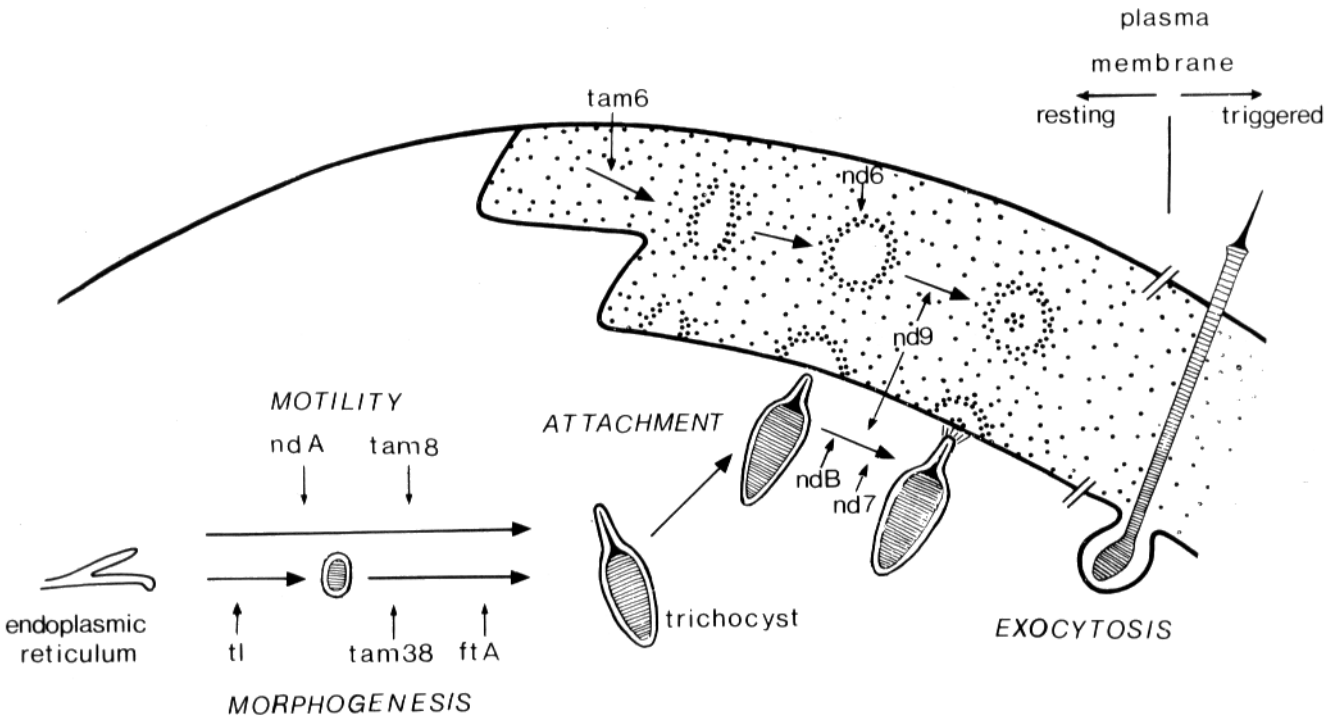


FIGURE 20 Reconstruction of the trichocyst pathway, as deduced from the properties of 10 mutants analyzed in this and other studies. The short arrows identify here the apparent site of block of the mutations. The scheme is not drawn to scale; the mature trichocyst is ~5 μm long, whereas the ring of particles in the plasma membrane is 300 nm in diameter.

TABLE IV

Summary of the Data Obtained on 10 Trichocysts Mutants

	Trichocyst					Plasma membrane				Cellular phenotype repaired by	
Mutant strain	Morphology	Motility	Attachment	Annulus	Function*	Parenthesis	Ring	Rosette	Ca^{++}ATPase‡	Microinjection	Conjugation
nd6	N	+	+	+	+	+	abn	−	−	−	+
nd7	N	+	+	+	−	−	+	abn/−	+/−	−	−
tam6	N	+	−/+§	+/−	+	abn	N	−/+		−	+
tam38	abn	+	−	−	−	N‖	−	−	−	−	−
nd9 27°C	N	+	+	+	+	+	+	−	−	+	+
nd9 18°C	N	+	+	+	+	+	+	+	+		
tam8	N	−	−	−	−	+	−	−	−	−	−
tl	−	...	...	...	...	+	−	−	−	−**	
ftA	abn	+	−		−					−	
ndA	N	−	−		−					−	
ndB	N	+	+		−					−	

The data on *nd9*, *tam8*, *tl*, *ftA*, *ndA*, and *ndB* are taken from Beisson et al. (6) and Aufderheide (2).

N/abn represent normal vs. abnormal trichocyst morphology or plasma membrane array configuration.

* The function is + when the mutant trichocysts can attach and be excreted in a *ftA* recipient cell.

‡ Presence or absence of the cytochemically identified Ca^{++}-ATPase activity at trichocyst attachment site. Data from Plattner et al. (24).

§ The +/− indicates the presence or absence of a particular feature, or in the case of microinjection and conjugation experiments, the repair or absence of repair.

‖ The parentheses in the mutant *tam38* are well organized and classified provisionally as N; however, the significance of the "double" parentheses remains obscure (see Results).

** K. Aufderheide, unpublished observations.

tion has still been found that blocks this process. In contrast, assembly of the rosette and exocytotic ability require specific properties of the trichocyst compartment (*nd7*), of the cytoplasm (*nd9*), and of the plasma membrane (*nd6*).

Although their function remains unknown, we believe that trichocysts provide an excellent model system for the analysis of exocytosis of secretory vesicles because it is the only system currently available that allows the combined use of genetic, physiological, and cytological approaches. It is, therefore, interesting to discuss briefly the consistency of our data with results obtained in other systems and to point out what original information of possibly general significance is provided by our results.

In yeast, numerous mutants deficient for acid phosphatase

secretion have been isolated (20). The genetic control of this secretory pathway appears as complex as in *Paramecium*, 23 loci have been identified as compared with at least 24 in Paramecium (11). Furthermore, the data on yeast suggest that the mutations would affect different steps (development of the secretory vesicles, transport, exocytosis), as in the case of trichocysts.

In another secretory system, the chromaffin granules, which has been mostly studied by biochemical and cytological methods, it has been shown that actin filaments are associated with the chromaffin granules (10) and that α-actinin is present in the granule membrane (17). The function of these actin filaments in the migration of the granules towards their exocytotic sites has been postulated (10). Similar studies on the known trichocyst motility mutants may be fruitful and it is worth pointing out that trichocyst motility and attachment to the cortex are cytochalasin B-sensitive (K. Aufderheide and J. Beisson, independent unpublished observations).

An important contribution of the trichocyst system is to demonstrate that interactions between trichocyst and plasma membrane are controlled by specific factors in both plasma membrane and secretory vesicles. It is, of course, implicitly evident that the various membrane fusion events that take place within a cell (secretion, fertilization, lysosomal cycle, etc) need to be under precise and differential control. However, in no other system than the trichocyst system has it yet been demonstrated that specific membranous factors (proteins?) do control localized interactions between membranes. Finally, biochemical (12, 13) and cytological (4) studies on chromaffin vesicle exocytosis suggests that membrane fusion would require some connecting material between plasma membrane and the membrane of the secretory vesicles. This material would help promote membrane fusion, possibly by bringing the two membranes into closer contract upon stimulation (increase in Ca^{++} concentration?). On the basis of purely physiological studies on the mutant *nd9* (6), the existence of potential connecting material has been demonstrated in *P. tetraurelia.* Such a connecting material might be a common feature involved in membrane fusion processes and might contribute to controlling membrane fusion by its differential sensitivity to microenvironmental conditions.

In conclusion, the data on the trichocyst system show that cellular control of exocytosis apparently involves the cooperative participation of many specific gene products, some of them in the trichocyst itself and others in the cytoplasm and the plasma membrane. The analysis of the trichocyst system is now amenable to biochemical analysis aiming at the characterization of the membranous and cytoplasmic proteins involved in this control.

This work was supported by the Ligue Nationale Francaise Contre le Cancer and by grant 7770267 from the Délegation Génerale à la Recherche Scientifique et Technique.

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Received for publication 3 June 1980, and in revised form 18 September 1980.

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