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Short Communication

What Contributes to Daughter Cells Separation during Cytokinesis of *Amoeba proteus*

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Summary. Before the furrow formation the non-polar general contraction resulting in cell de-adhesion and spherulation, and the following relaxation leading to re-adhesion, flattening and spreading, are both necessary prerequisites of a successful cytokinesis in *A. proteus.* The bipartition begins by sudden generation of two divergent cytoplasmic streamings at the late anaphase, always before the formation of furrow. All these facts fit better with the polar relaxation model than with the equatorial contraction model of initiating the fission of amoeba. After formation of the furrow the contractile ring gradually constricts the cytoplasmic connection between daughter cells. Endopasm flow in the connection bridge is no more bipolar but irregularly reversing; it compensates hydrostatic pressure differences between daughter cells. The final break of the connection is explained by its stretching because of the disparate locomotor activities on both sides of the furrow and owing to the cytoskeleton disassembly inside the connecting bridge.

Key words: *Amoeba proteus,* cell adhesion, cell motility, cytokinesis.

INTRODUCTION

The large majority of Protists have a well pronounced motor polarity expressed by differentiation of anterior and posterior body poles. This poses a problem of the mode of cell division. The fission either may be perpendicular to the body axis (as in ciliates), or longitudinal (as usual in flagellates). Amoebae have resolved this problem differently, by the loss of motor polarity and a transient halt of locomotion. *Amoeba proteus* ceases moving, contracts and rounds up prior to cytokinesis (cf. Figs 1, 4). However, motility of the two future daughter cells is restored long before they definitely separate. It raises the question whether this final step of cytokinesis is still effected by constriction forces originating locally in the division furrow, as in metazoan eggs and non-motile cells, or is it completed by traction forces generated at some distance from the two sides of the furrow, i.e., by both prospective daughter cells tending to move in different directions. This second mechanism should normally necessitate a bipolar adhesion to the substratum to pull the mother cell in two.

As a matter of fact, in the earliest experimental studies of the cytokinesis of *A. proteus* (e.g. Chalkley 1935, 1951) the pulling force resulting from locomotion of daughter cells was considered as the principal factor

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of the cell division in amoeba. The last study of cytokinesis in this species (Rappaport and Rappaport 1986) concluded, in contrast, that "the constriction activity in the furrow region resembles that of metazoan cells...", whereas "attachment and locomotion... were not essential for cytokinesis, and their involvement is restricted to the final parting of the cytoplasmic thread that connects the daughter cells." These statements are probably partly correct, however they need re-examination, because observations of amoebae strongly flattened under a layer of Halocarbon oil, made by Rappaport and Rappaport (1986), are not conclusive as far as the role of attachment to the substratum and of the natural flattening and spreading of cells at the early stages of cytokinesis are concerned.

In our experiments amoebae were examined in chambers about 100 μm deep, free to attach or not, and to spherulate or spread on the substratum. Besides recording the course of cytokinesis in living specimens in a differential interference contrast microscope (DIC), the presence and distribution of adhesive organelles: minipodia and rosette contacts described recently by us (Grębecki *et al.* 2001), were examined in a scanning electron microscope (SEM) in amoebae fixed at different stages of division, and the accumulation of F-actin in the division furrow was demonstrated in a confocal laser scanning microscope (CLSM) after fluorescent phalloidin staining.

MATERIALS AND METHODS

Amoeba proteus cells (strain C_L), were grown at 20±1°C in glass culture dishes with Pringsheim medium. They were fed twice a week with *Tetrahymena pyriformis* and the division spheres were collected one day after feeding. The observation chambers were bordered with parafilm strips which kept the cover slip about 100 μm over the slide, thus allowing the dividing amoebae freely attach to or detach from the substratum. The course of cytokinesis was recorded *in vivo* in a Biolar microscope (PZO, Warsaw) equipped with DIC optics of Pluta system and coupled with a C2400 Hamamatsu camera and NV8051 Panasonic time-lapse recorder adjusted to 8x time compression.

The samples for SEM were fixed in 3.5% paraformaldehyde with 0.5% acrolein, dehydrated through a graded series of ethanol and acetone, dried by the CO_2 critical point method, and coated with carbon and gold. They were examined in a Jeol 1200 EX transmission electron microscope with an ASID 19 scanning attachment, operating at 80 KV. Other samples, after the same fixation, were stained with 1% phalloidin labelled with fluorescein isothiocyanate (Sigma, St. Louis) and examined for F-actin in an Olympus FV-500 confocal laser scanning microscope.

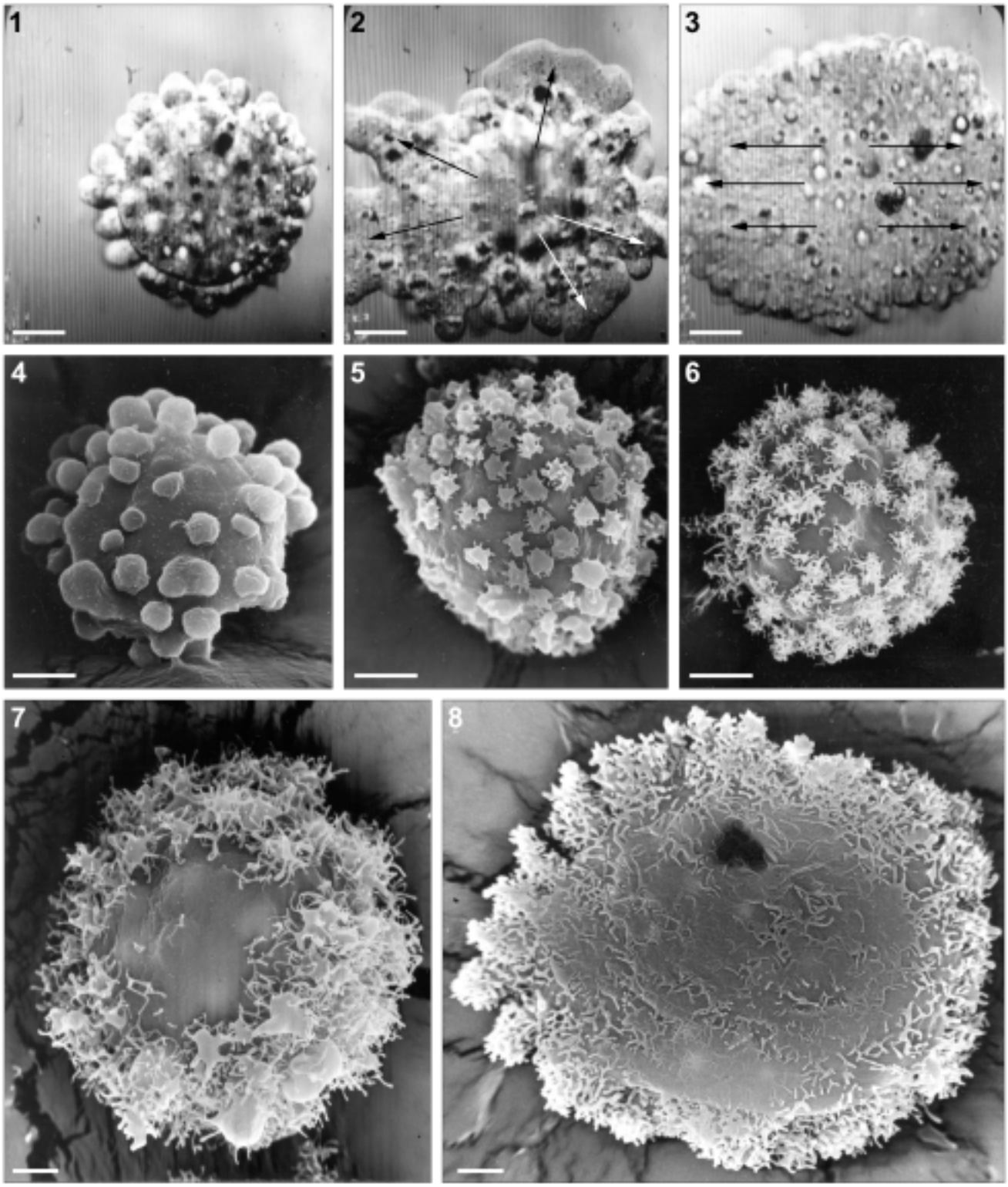
RESULTS AND DISCUSSION

The division spheres formed in prophase (Fig. 1) freely float in the medium, or so loosely contact with the substratum that they become easily detached by any slight flow of the culture medium, or spontaneously. This is consistent with the absence of any adhesive organelles (such as minipodia) on their surface examined by SEM (Fig. 4). There is no endoplasmic flow inside the spheres, but the cytoplasmic inclusions (seen in Figs 1, 9) show intense erratic movements, independently one from another and never form at least locally co-ordinated streamings. It proves that at this stage the cell is incapable of building any hydrostatic pressure gradient, and means that the usual polarity of peripheral contraction has been lost.

In fact, the circular contour of the cell body appeared in DIC *in vivo* (Figs 1, 9) as a layer much more optically dense than in the locomoting amoebae. In the fixed division spheres stained with FITC-phalloidin CLSM revealed (Fig. 10) accumulation of F-actin in the cortical zone (and in the perinuclear region). Some division spheres, fixed for SEM, were mechanically injured with a microneedle before coating with carbon and gold; in many cases that exposed their submembrane structure to view (Fig. 12). This method always revealed a very extensive development of the three-dimensional network of microfilaments under the surface of division spheres (Fig. 13), which in high magnification (Fig. 14) look identical to F-actin meshworks frequently demonstrated in the literature.

Contraction of this cortical network produces tightly packed bulbous protuberances on the surface of division spheres. These protuberances, however, neither are homologous to the blebs nor to the caps of locomotor pseudopods of moving amoebae and of some motile tissue cells which contain optically empty hyaloplasm (cf. for example Grębecki 1990, Keller and Eggli 1998). The protrusions of the division spheres are, in contrast, full of cytoplasmic inclusions (Fig. 9) and are surrounded by F-actin layer (Fig. 11).

The excessive accumulation of F-actin in the cell cortex, uniform squeezing of protuberances around the whole surface, lack of hydrostatic pressure gradients inside, absence of adhesive organelles and deficiency of attachment, all-together strongly suggest that the cell division of amoeba is preceded by a supernormal and uniform cortical contraction. It fits well with the view that not the equatorial over-contraction, but the



Figs 1-3. The same cell of *Amoeba proteus* recorded in DIC from the prophase to the late anaphase. **1** - contracted, non-adhering division sphere without endoplasmic flow; **2** - centrifugal streamings and arisal of adhesive pseudopods (arrows) lead to cell spreading; **3** - bipolar flow (arrows) elongates the cell to elliptic shape. Scale bars - 20 μm

Figs 4-8. Minipodia and rosette contacts on the surface of prophase-to-anaphase cells examined in SEM. **4** - absence of adhesive organelles in an early division sphere (corresponds with Fig. 1); **5, 6** - development of minipodia and rosette contacts; **7** - marginal re-arrangement of adhesive organelles during cell spreading; 8 - adhesive organelles at the stage of bipolar streaming and cell elongation (corresponds wi th Fig. 3). Scale bars - 20 μm

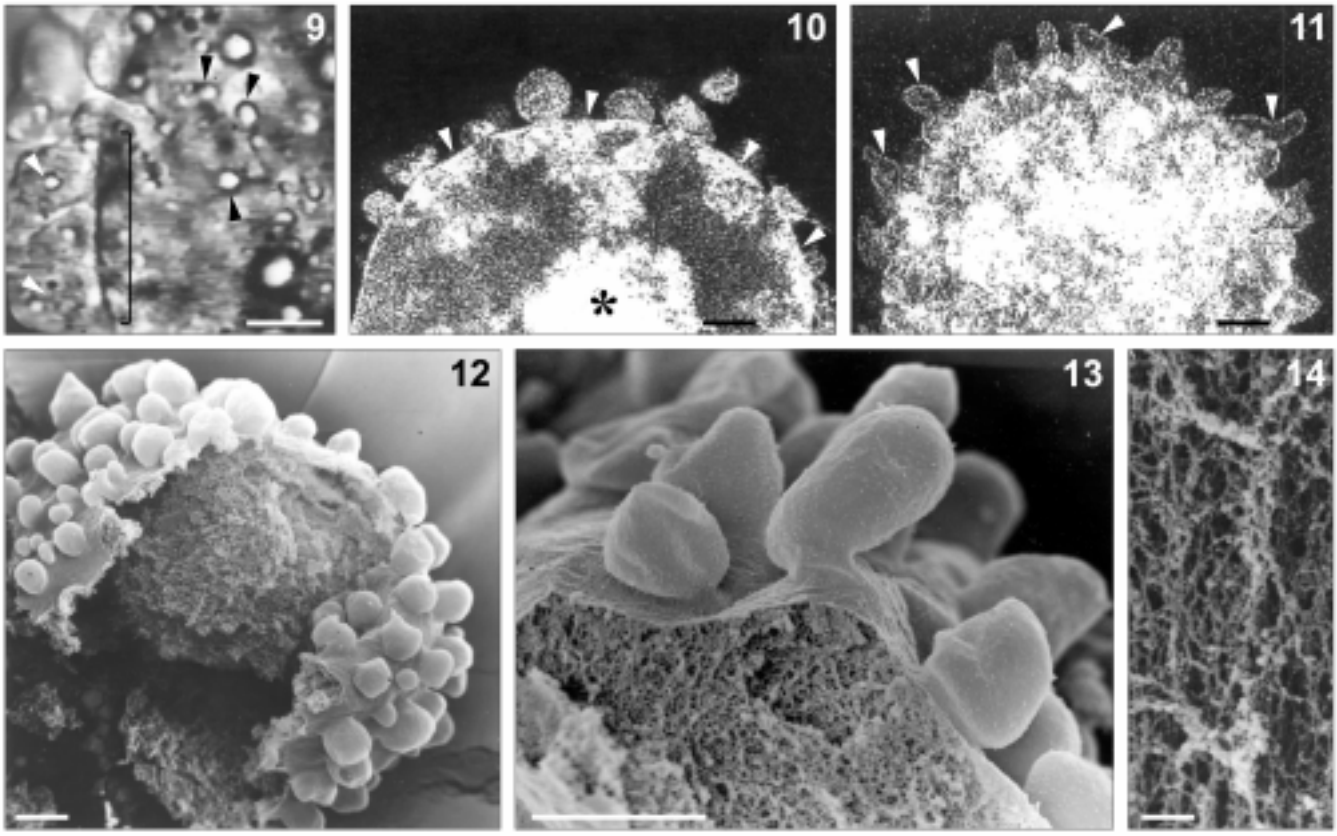
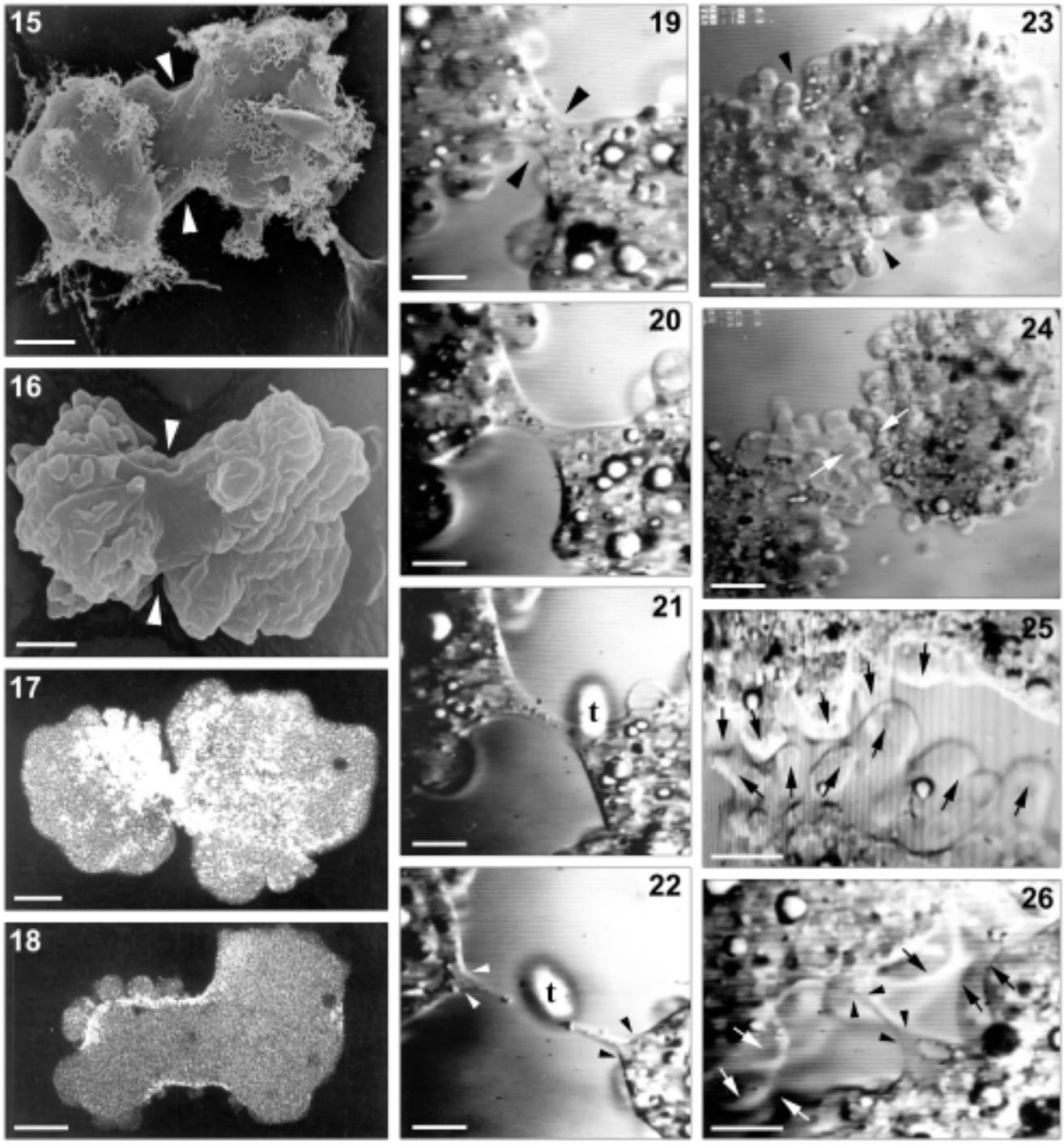


Fig. 9. High magnification of a fragment of division sphere in DIC (corresponding with Fig. 1); note elevated optical density of cell cortex (brace), and cytoplasmic particles in the cell body (black arrowheads at right) and in bulbous protuberances of the surface (white arrowheads at left). Scale bar - 10 μm
Figs 10, 11. F-actin staining with FITC-phalloidin in division spheres examined in CLSM; **10** - note F-actin accumulation in the cortex (arrowheads) and around the nucleus (asterisk); **11** - actin sheets around surface protuberances. Scale bars - 10 μm
Figs 12-14. Extensive sheet of cortical microfilaments seen in SEM in the division spheres injured with a microneedle. **12** - general view of an injured specimen; **13** - the microfilaments layer beneath the cell surface; **14** - high magnification of the microfilaments network. Scale bars - 10 μm in 12, 13, and 2 μm in 14.

polar relaxation is needed to start cytokinesis. This concept, repeatedly appearing in the past (e.g. Chalkley 1935, Wolpert 1960) as well as more recently (e.g. White and Borisy 1983, Bray and White 1989, Gręboecki 1994), seems to be especially well applicable to the case of *A. proteus* in which the whole contractile cortex creates tension, but the locomotion is initiated and controlled by frontal relaxation (Gręboecki 1981, 1990, 1994).

The next event, beginning probably in the late metaphase, is the reconstruction of adhesive organelles. SEM reveals that the bulbous protuberances of the division spheres start producing microextensions (Fig. 5) which gradually achieve (Fig. 6) the size and shape of minipodia grouped in adhesive rosettes (identical with those described by us in the attached and moving interphasal amoebae: Gręboecki *et al.* 2001). It is worthy to note that Sanger and Sanger (1980) described the disappearence and reconstruction of "microvilli", similar to our "minipodia", during cytokinesis of PtK_2 cells of epithelial origin.

After this stage, in amoebae recorded *in vivo*, the chaotic particle movements become suddenly (within 1-2 min) ordered into a few local centrifugal streamings, and several flat lobose pseudopodia appear and spread over the glass (Fig. 2). This is the typical behaviour of *A. proteus* re-adhering to the substratum (Kołodziejczyk *et al.* 1995). Re-adhesion leads to cell spreading, so that the division spheres become flat disks (compare the optical cross-sections of the same amoeba in Figs 1 and 2). Examination in SEM shows at this stage minipodia and rosette contacts arranged along disk edges (Fig. 7). As we have suggested earlier (Gręboecki *et al.* 2001), marginally distributed adhesive organelles contribute to further cell spreading and flattening (compare Fig. 7



Figs 15, 16. SEM pictures of amoebae during formation of the furrow (arrowheads). **15** - the usual bipolar distribution of minipodia and rosette contacts; **16** - the exceptional case of furrowing amoeba without any adhesive organelles in view. Scale bars - 20 μm
Figs 17, 18. CLSM pictures of amoebae stained with FITC-phalloidin during formation of the furrow. **17** - general view of F-actin aggregation toward the furrow; **18** - thin longitudinal optical section through the furrow shows peripheral actin; bodies of the future daughter cells are not seen (they were out of the section plane). Scale bars - 20 μm in 17, and 10 μm in 18.
Figs 19-22. DIC records of the separation of substratum-attached daughter cells from the stage of a deep furrow (arrowheads in **19**), through gradual narrowing of the connecting bridge (**20, 21**) up to its final break out (between arrowheads in **22**); t - is a *Tetrahymena* which accidentally entered into the field. Scale bars - 20 μm
Figs 23-26. DIC records of amoebae dividing without attachment to the substratum. **23** - the furrow is masked by pseudopods (between arrowheads); **24, 25** - general view and a higher magnification of pushing one daughter cell against another by growing pseudopods (arrows); **26** - a connecting bridge (between small arrowheads) distended by the neighbour pseudopods (arrows). Scale bars - 20 μm

with 8). The three DIC records of the same cell shown in Figs 1-3 demonstrate that its cross-sectional area increased about 3.5 times. In this respect the sequence of Figs 4-8 looks equally impressive, but it is less exact, since it is composed of different specimens which might originally vary in size.

Summarizing all what happens before the furrow formation, we conclude that the non-polar general contraction resulting in cell detachment and spherulation, as well as the following relaxation leading to cell re-adhesion, flattening and spreading, are both necessary prerequisites of a successful cytokinesis in *A. proteus.*

The bipartition of the mother cell begins in amoeba by a dramatic change of intracellular streaming pattern at the late anaphase. Suddenly, the disparate centrifugal endoplasmic streamings become strictly bipolar and the round disks quickly turn into the oval shape (Figs 3, 8). We should strongly stress that two steady streamings oriented exactly in two opposite directions occur, during the whole cytokinesis of *A. proteus,* only at this brief moment, and that this happens before the formation of furrow. Again, it fits better with the polar relaxation model than with the equatorial contraction model of initiating the fission of amoeba.

The time-lapse records in DIC show that during formation of the furrow both divided cell halves generate streamings and extend or retract pseudopods in all directions, and the motor activity of each of them is independent of another. In the furrow region the endoplasm does not steadily flow in two opposite directions from the middle, as it should be expected, but performs shuttle movements reversing at irregular periods. It means that this flow compensates the oscillating pressure differences between both future daughter cells, which are greater than the pressure gradient created by contraction in the furrow.

The motor activity of the prospective daughter cells is consistent with the observation that during formation of the furrow almost all amoebae remain attached to the substratum, and with the demonstration by SEM (Fig. 15) that adhesive minipodia and rosette contacts are common at both opposite cell halves, but scarce or absent at the furrow region. In whole our material we found just one case of amoeba devoid of adhesive organelles during furrow formation (Fig. 16). *Dictyostelium discoideum* may divide even in suspension, but if its cytokinesis is examined by reflection interference microscopy on a solid surface the cell-substratum contact also appears most extensive and stable under the future daughter cells (see pictures in: Weber *et al.* 1999). If fibroblasts are in this respect similar to amoebae, then the wrinkling of silicone elastic substratum during their division, which was presented as a specific result of the contraction in the furrow (Burton and Taylor 1997), could rather result from the traction exerted between the adhesion sites under the bodies of both future cells.

The equatorial constriction certainly results from actomyosin contraction in the furrow. In the cortex of amoebae, however, a contraction always leads to gradual disintegration of the F-actin network (contraction-solation coupling: Taylor and Fechheimer 1982). Therefore, the outflow of depolymerized actin from the furrow must be compensated by the centripetal F-actin import along the cell periphery (cortical flow theories: Bray and White 1989, Grębecki 1994). Such cortical transport of F-actin and myosin II toward the furrow was well studied in the dividing *Dictyostelium* amoebae (Kitanishi-Yumura and Fukui 1989, Fukui and Inoué 1991, Yumura and Fukui 1998, Yumura 2001). Strangely, the presence of F-actin in the division furrow of *A. proteus*, although it is obviously expectable, has never been shown. Its aggregation toward the furrow is demonstrated in Fig. 17, and a thin optical section running exactly along the furrow region is presented in Fig. 18.

After formation of the furrow and before final separation (Figs 19-22) the dividing amoebae usually were still attached to the substratum. We have found in whole our material recorded *in vivo* over 50 cases of cytokinesis completed by the adhering cells, but only 4 cases of division accomplished by amoebae which have spontaneously de-adhered. Possibly, the furrowing amoeba without adhesive organelles found in SEM (Fig. 16) belonged to the same category of cells. Moreover, the non-attached amoebae needed up to 1 hour to disconnect, while the well adhering ones achieved it in the limits of 10-20 minutes. Without adhesion both future daughter cells are spherical and extend short pseudopods in all directions and in the three dimensions (Figs 23, 24). Some of these pseudopods produced by the two cells may meet and push against each other (Fig. 25) and then mechanically disrupt the slender connecting bridge situated between them (Fig. 26). This mechanism, proposed very long ago (Chalkley 1935, Liesche 1938), sufficiently explains how the dividing amoebae experimentally detached from the substratum (Rappaport and Rappaport 1986) or spontaneously de-adhering (the present observations) can complete cytokinesis by

separation in two. It must not be, however, forgotten that in *A. proteus* the cell division without adhesion is exceptional.

Normally the daughter cells of *A. proteus,* still linked by a cytoplasmic strand, firmly adhere to the substratum and try to move in various directions. These are random movements not co-ordinated between the two future amoebae. Their exact opposition capable of breaking the connecting bridge up (as in Figs 19-22) can be achieved only by chance, and it may take several minutes until it happens. In contrast to some early ideas (constriction ring as "tail organizer" for the new cells: Goldacre and Lorch 1950), the furrow region changing into a connecting bridge is never wrinkled as the tail of an interphasal amoeba, but smooth and apparently stretched by daughter cells moving farther and farther apart (Figs 19-21). Breaking up of the connection is immediately followed by the elastic recoil of both parts, proving that the bridge was not contracting but distended by pulling in opposite directions (compare Fig. 21 with 22).

At least three factors may contribute to the initial constriction and the final rupture of the bridge which connects the two parts of a dividing *A. proteus*: (1) function of the contractile ring in the furrow, (2) stretching the connecting bridge by opposite locomotor activities on both sides of the furrow, (3) cytoskeleton disassembly inside the connecting bridge.

(1) The contractile ring certainly plays a major role in the first phase by gradually narrowing the lumen of the connection, but it cannot cut it in two by constriction, since it acts from the inside, not from the outside of this cytoplasmic tube; this argument applies to amoebae as well as to all other motile and non-motile cells.

(2) At the final separation phase the connection is broken, in amoebae, by independent motor activities of future daughter cells, *i.e.*, by pulling when they move on a solid substratum, or by pushing one against another when they are in suspension; this mechanism may operate in the cells capable of amoeboid movements, but not in non-motile cells.

(3) The coupling of contraction with solation and the disintegration of actomyosin network in moving amoebae, which were mentioned above, may lead with the elapse of time to exhaustion of the cytoskeletal material in the connecting bridge, what should help or provoke breaking up this last link between daughter cells; gradual disassembly of the contractile ring was observed during cleavage of sea urchin eggs (Schroeder 1972) and in dividing HeLa cells (Maupin and Pollard 1986); this factor may probably be significant in all types of motile and non-motile cells.

For the time being the speculations about factors of the final separation of daughter cells in *A. proteus* may be in the best way concluded by quotation of the words of Robinson and Spudich (2000) that "the molecular control of this late step is only beginning to be uncovered and promises to bring many more surprises."

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