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Gravireception and Graviresponses in Ciliates

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Summary. An account is given of approaches to gravireception, terminology, mechanisms of responses to gravity as investigated and documented in the literature, and sensorimotor coupling properties in ciliates. Current theories and methods are discussed, and previously published experimental data on graviresponses are reviewed.

Key words. Gravireception, gravitaxis, gravikinesis, ciliates, electrophysiology, sensorimotor coupling

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1. INTRODUCTION

Gravireception in protists is an area of so far limited knowledge and understanding. A wealth of data, in particular of *Paramecium*, has been accumulated in the past century, but there is not much common agreement on the mechanisms of gravitaxis. The reason for this is closely associated with the nature of gravity which rules the universe across billions of light years and affects small living things on Earth in a subtle manner. Experiments using gravistimulation are difficult to design and to analyze because gravity is not easy to manipulate. Moreover, gravistimulation persists. Unlike a mechanical stimulus, which induces sensory transduction, in the first line, by changing intensity (e.g., the magnitude of deformation of a structure), gravity maintains its amplitude under common circumstances. Sensory structures in animals and in plants have evolved, which specialize in gravireception and guidance of gravity-related behaviour.

An archetype of a gravireceptor is the statocyst[1] (Markl 1974, Vinnikov 1974). In an extracellular cavity of this organ, a body of higher density, a statolith[1], is surrounded by (ideally) a globular shell of sensors, which may be represented by an epithelium or by intracellular organelles. The statolith exerts a constant load on a "lower" (or centrifugally placed) sensitive structure. Information about the organism's orientation in space arises from persistent stimulation and topology: the arrangement of the statocyst inside the animal, and the site of activation of the sensor via statolith inside the statocyst. The present review, in analyzing recent experimental data on behavioural responses of ciliates to gravity, assumes that the statocyst principle applies to gravisensation in protists, as it does in some algae and in the statocytes of roots in higher plants (for reviews see: Audus 1979, Björkman 1988, Sack 1991), although different signalling pathways may be employed in special cases. Comprehensive overviews of the effects of gravity on unicellular microorganisms have been published elsewhere (Haupt 1962, Roberts 1970, Machemer and de Peyer 1977, Sievers and Volkmann 1979, Bean 1984).

2. APPROACHES TO GRAVIRECEPTION

2.1 ENERGETIC CONSIDERATIONS

A fundamental requirement for gravireception is that a minimal stimulus energy can be supplied to the organism in the gravitransduction process. This energy (or work to be done) is approximated for a ciliate assuming a cylindrical body of 200 µm length and of 40 µm diameter. With a volume of 2.5×10^{-7} cm^3, and a density of 1.04 g/cm^3 (*Paramecium*: Koehler 1922, Fetter 1926, Taneda 1987, Kuroda and Kamiya 1989), the cytoplasm exceeds the mass of fresh water by 1.008×10^{-8} g and exerts a force of 10^{-10} N (10^{-5} dyne) to the lower membrane. In a vertically swimming *Paramecium*, this force corresponds to an extra pressure of about 0.1 Pa (8×10^{-4} N/cm^2), which is reduced to about 1/6 in a horizontally swimming cell due to the larger area of the lower membrane. Can this small value of pressure give rise to a biological response? Comparison with gravitropic responses in plants shows that this is possible. In the green alga *Chara* a pressure increment of 10^{-4} Pa (Hejnowicz et al. 1985) and gravity-induced changes in shear stress in the plasma membrane modulate cytoplasmic streaming (Sievers et al. 1991), and a pressure difference of about 0.3 Pa thought to be exerted by starch grains (amyloplasts) in the statocytes of roots in higher plants induces positive gravitropic growth (Volkmann 1974).

Apart from comparison with other gravisensitive systems, an adequate energy of a stimulus for generation of a signal by the cell must exceed the energy level of thermal noise (2×10^{-21} J). The minimal energy to induce gravitransduction is estimated at 3×10^{-20} J (Björkman 1988), corresponding to a force of 3×10^{-11} N acting over a distance of 1 nm. In *Paramecium*, the cytoplasm-water density difference allows the available force of 10^{-10} N to deform the "lower" membrane by an unknown amount. If it is tentatively assumed that this force moves a gravity sensor over a path length of 1 nm, in analogy to effective deformation of an insect mechanoreceptor (Thurm et al. 1983), the work done will be 10^{-19} J and thus exceeds the energy of thermal noise by a factor of 50. Larger displacements of a membrane-integrated sensor give an even more favourable level of energy available for transduction.

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[1] Definitions of "statolith" vary in the literature. In the field of Zoology, it designates an extracellular body and is synonymous to otolith (Eckert and Randall 1983). In Botany, statoliths designate sedimenting masses inside statocytes (Sievers et al. 1991). For protozoans, we use the term "statocyst-type organelle" (or statocystoid; Bräucker et al. 1992) with respect to the common principle of function.

Energetic approximations show that gravireception in cells involves highly sensitive receptor mechanisms. Amyloplasts, which act as statoliths inside statocytes of higher plants, confer an energy of 5 to $10x10^{-19}$ J to the receptor assuming an effective sedimentation of 10 µm (Björkman 1988, Volkmann and Sievers 1992). Such energy corresponds to that of a single photon in the visible range of the electromagnetic spectrum. Gravireception is not the most extreme case of sensitivity in mechanotransduction. In auditory hair cells of the inner ear, the presumed sensitivity of the receptor ($6x10^{-21}$ J) approaches the level of thermal noise presumably due to probabilistic, that is, nondeterministic signal processing in transduction of sound (Hudspeth 1985). Comparison with effective pressures reported for stretch-activation of channels in prokaryotes ($2x10^3$ Pa; Martinac et al. 1987) and eukaryotes ($2x10^3$ to 10^4 Pa; Falke et al. 1988, Methfessel et al. 1986) shows that cells are four to five orders of magnitude more sensitive to gravity than to osmotic pressure, and that tangential stretching of channels is an unlikely mechanism of gravireception.

2.2 STRUCTURES SUITABLE FOR GRAVIRECEPTION

An obvious strategy would be the identification of statocyst-type[1] structures in cells (statocystoids). Loxodid ciliates, the marine *Remanella* and the limnic *Loxodes*, bear vesicles (Müller vesicles) with inclusions of $BaSO_4$ suggesting a statocyst function (Hubert et al. 1975, Rieder et al. 1982, Fenchel and Finlay 1986a). Similar Ba^{2+}- and Sr^{2+}-containing statoliths have been identified inside the gravitropic rhizoid of the alga *Chara* (Schröter et al. 1975, Sievers and Schmitz 1982). The Müller vesicle was carefully investigated by Fenchel and Finlay (1986a). The number of vesicles is proportional to the area of the surface membrane. In *Loxodes striatus* the Müller vesicles have a diameter of 7 µm. A membrane-limited spherical body of 3 µm diameter made up of 70% $BaSO_4$ connects to the extra kinetosome of a modified cilium by a string of microtubules, which extend from a postciliary ribbon. The statolith and connecting ribbon are intracellular components lined by an extra membrane, which protrude into the Müller vesicle in the manner of

a pendulum. Cell-orientation dependent positions of the statolith inside the Müller vesicle have been documented.

Several arguments in support of a statocyst-type function of the Müller vesicle have been put forward by Fenchel and Finlay (1984, 1986a): (1) The passive settling rate of the statolith in *Loxodes* (17 µm/s) exceeds velocities of Brownian motion ($6x10^{-2}$ µm/s). (2) In the marine *Remanella* a 4µm-statolith is made up of the lighter $SrSO_4$ (density: 3.9 as compared to 4.5 g/cm^3 in $BaSO_4$) so that the statolith size can compensate for the more unfavourable density of the mineral (and of the salinity of the vesicle). (3) Distortion of the base of the connecting cilium may activate mechanically sensitive channels to modulate the membrane potential in agreement with principles in ciliate mechanosensitivity (Machemer and Deitmer 1985).

Calculation of the settling force of the statolith in a Müller vesicle of *Loxodes* ($4.9x10^{-13}$ N) suggests that the work done by the statolith during a 2µm-excursion from the neutral position (10^{-18} J) is well above the noise level of Brownian motion. Such safety margin for gravitransduction compares favourably with the low density of the cytoplasm in *Loxodes* (≤1.02 g/cm^3; unpublished observations), which exerts a critically small or even subthreshold gravitational force on the lower membrane of the cell ($3x10^{-11}$ N). Both structural and energetic considerations of gravitransduction suggest a need for focusing the natural gravity stimulus toward a very limited number of highly sensitive receptor sites.

2.3 BEHAVIOURAL ANALYSIS

If gravity is a meaningful stimulus for a microorganism, we expect that behaviour reflects transduction. Chemical, photic and mechanical stimuli modulate the velocity and orientation of swimming, and hence the accumulation or dispersal in ciliates (for reviews see: Machemer 1988b, 1989; Van Houten 1992; Machemer and Teunis 1993). Gravitactic (formerly: "geotactic") responses have been documented and analyzed previously. Establishing unequivocal evidence of a physiologically guided graviresponse in unicellular organisms implies gravireception. Conversely, a negative evidence, that is, documentation of the absence of such behaviour would be a case against gravitransduction.

3. TERMINOLOGY

"Gravitaxis" is commonly used to describe behavioural responses to the gravity vector. Because in ciliates gravity can affect the rate of locomotion (gravikinesis) and orientation (gravitaxis), it may be pragmatic and corresponding to practice in other fields of sensory physiology to classify orientational and kinetic responses under the generalizing title of "graviresponses". We use graviresponses as a descriptive term, which does not discriminate between behaviour caused by physiological or non-physiological mechanisms. Such term is useful to characterize behaviour because, under terrestrial gravity, active responses and passive reactions may occur at the same time and, under circumstances, be difficult to separate experimentally. The term of graviresponses is sufficiently generalized to cover also gravity-induced orientation or growth responses of algae and higher plants (gravitropism).

The definition of gravitaxis remains, even with these restrictions, ambiguous depending on the level of analysis. A single cell shows gravitaxis upon *reorientation* from, e. g., downward to upward swimming maintaining its observed velocity. Behaviourally, this would be a case of a genuine orientational response. If, however, the sedimentation rate of the same cell is taken into account, the rate of active downward propulsion was less than the rate of active upward propulsion. The observed constant swimming rate resulted from (1) the invariant basic swimming rate, (2) a gravikinetic increment and (3) sedimentation; hence, the observed graviresponse includes a kinesis. A behavioural difference may also exist between a single cell switching between upward and downward orientations, and a population of cells which does not drift in a vertical direction because of alternating reorientations of individuals. For such case, the term of neutral gravitaxis has been proposed (Machemer-Röhnisch et al. 1993).

According to Fraenkel and Gunn (1940), a kinesis signifies activation of locomotion by the strength of a stimulus, ignoring its direction. An increase of the response with rising stimulus amplitude was termed "direct kinesis", a reciprocal relationship "inverse kinesis" (Kennedy 1945a, b). Unfortunately, these relationships are also described in terms of "positive kinesis" and "negative kinesis" (Pfeffer 1904, Diehn et

al. 1977), that is, using for criterion the response rather than the stimulus.

A gravikinesis identified in *Paramecium* (Machemer et al. 1991, Ooya et al. 1992) does not correspond to conventions because it is affected by the strength as well as by the direction of the gravity vector (Machemer-Röhnisch et al. 1993). In analogy to definitions of a taxis with respect to the direction of the stimulus source, we use the terms of "positive" and "negative gravikinesis"[2]. A negative gravikinesis of, for instance, *Paramecium* and *Loxodes* is a gravity-induced increase in active locomotion of upward oriented cells, and decrease in active locomotion of downward oriented cells. An orienting mechanism, passive or active, is causal for the sign of a gravikinesis, which does not produce orientation. Indirectly, a gravikinesis can contribute to accumulations of negatively gravitactic cells (see 8.3.10.6). It is seen that a gravikinesis is intermediate between the definitions of taxis and kinesis.

4. MECHANISMS IN GRAVIRESPONSES

The study of graviresponses in ciliates is difficult because these responses may result from action of more than a single mechanism. An overview of previous analyses shows that for the isolation of physiological graviresponses in ciliates, and for approaches to identification of the signalling chain, experimental strategies must take into account a variety of parameters which potentially affect the behaviour of cells in the gravity field.

4.1 STATIC HYPOTHESIS (GRAVITY-BUOYANCY MODEL; VERWORN 1889)

This hypothesis explains the negative gravitaxis of *Paramecium* on the assumption that the centre of gravity does not coincide with the geometric centre of the cell. If the centre of gravity is shifted posteriorly, as suggested by the wider diameter of the posterior half of the cell, randomly oriented cells will end up swimming forward with their anterior ends upward. A similar explanation was applied to negative gravitaxis in the green flagellate *Euglena* (Wager 1911), which may have a *Paramecium*-type shape. Kuźnicki (1968) crucially tested the static

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[2] An interesting alternative suggestion: "parallel gravikinesis" - "antiparallel gravikinesis" (W. Haupt, personal communication) would have to be defined so as to account for possible angular offsets from the direction of gravity.

hypothesis immobilizing *Paramecium* with NiCl₂. Sedimentation of *Paramecium caudatum* in vertical capillaries led to a major proportion of cells oriented with their anterior ends upwards, but in other species of *Paramecium* this was not confirmed. In well-fed immobilized cells of *P. caudatum* (wide rear ends) an anterior-end-up orientation was improved as compared to cells starved for 10 days. The effect of feeding state on orientation of Ni^{2+}-immobilized paramecia was confirmed by Taneda et al. (1987), who centrifuged cells in an isodensity solution (1.042 g/cm³) and found in ≤2 days old cultures 50% of the cells oriented vertically upward (±20°). The distribution was random in cells from ≥5 days old cultures. Fukui and Asai (1980) found preferred upward orientations in normally fed *Paramecium*, and this behaviour was even more pronounced after immobilization using Triton-X extraction. The evidence of a "posterior density bias" led the authors to develop equations from mechanical and hydrodynamic parameters predicting the curvature of traces of upward reorienting paramecia (Fukui and Asai 1985). All experiments show that the static hypothesis is applicable to appropriately fed cultures of *Paramecium*, but its predictive value is still much limited for individual cells.

4.2 HYDROSTATIC HYPOTHESIS (JENSEN 1893)

This earliest alternative model of negative gravitactic swimming assumes that *Paramecium* can detect the hydrostatic pressure gradient along a vertical water column. Physically, this is a quite reasonable view because the increment of absolute pressure corresponds to the force exerted by the mass of the cell over its horizontal cross-sectional area. This increment, 2.6 Pa for a vertically swimming *Paramecium*, exceeds the pressure derived from density differences by a factor of about 30 (see 2.1). It is nevertheless difficult to anticipate how "upper" and "lower" sensors (in the membrane?, cytoplasm?) can compare local pressures, which are 4 orders of magnitude smaller than the range of hydrostatic pressure in a 2m-vertical column of water (the typical range of natural habitat in a ciliate). In addition, experiments applying a pressure of 3.4×10^6 Pa (34 atm) to *Paramecium* lead to comparatively inconspicuous changes in swimming behaviour, that is, a 30% reduction in swimming velocity and a 60% reduction in frequency of spontaneous reversals (Otter and Salmon 1985).

Down-interpolation to 2.6 Pa leaves no room for generation of a signal (calculated change in velocity: 0.002%). In elasmobranch and mammalian mechanoreceptors, sensitivity of the membrane to strain (i.e. deformation) was electrophysiologically demonstrated, whereas stress (i.e. absolute pressure) was ineffective for transduction (Loewenstein 1960, 1965). The hydrostatic hypothesis was experimentally tested in *Paramecium* using the swimming rate and orientation for criteria. Schaefer (1922) did not see changes in swimming velocity at different altitudes inside a water column. Taneda (1987) reversed the hydrostatic pressure gradient and released the extra pressure in a step-type fashion. Tracking of individual cells in the column showed that negative gravitaxis was unaffected by these manipulations.

4.3 GENERAL STATOCYST HYPOTHESIS (LYON 1905)

Pressure gradients due to differences in density are common in eukaryote cells. The idea that a plant cell might sense the weight of its own cytoplasm to signal the gravity vector (Czapek 1895) was subsequently rejected on the grounds that the resulting pressure difference (10^{-5} N/cm² for a 10 μm cell) was too small for an adequate stimulus (Noll 1900, see paragraph 2.1 for comparison with more recent work). Lyon (1905), in applying the view of density-induced local pressure to *Paramecium*, postulated that the cell as a whole served as its own statocyst to perform gravitaxis. The author centrifuged *Paramecium* in tapering capillaries at room temperature and at 0°C and found equal proportions of upward and downward oriented cells, which disagreed with predictions of the buoyancy model (see 4.1). Koehler (1921, 1922) reconfirmed Lyon's findings: after feeding iron particles to *Paramecium*, the cells showed negative gravitaxis irrespective of the distribution of food vacuoles. "Iron-cells" swam away from the pole of a magnet ("magnetotaxis"), and their swimming rate was increased by 50% as compared to unstimulated cells (Koehler 1931). Specimens of *Paramecium*, which swam against the gravitational pull in the centrifuge ("centrotaxis"), showed a raised swimming rate after centrifugation (Koehler 1930). These data supported the statocyst hypothesis but failed to reject the static hypothesis altogether. Koehler (1930) acknowledged this unsatisfactory state in summarizing "that as long as the burning questions of (cell) excitation and conduction are still open, all conclusions relating to gravitaxis remain hypothetical".

4.4 RESISTANCE HYPOTHESIS (DAVENPORT 1908)

If a microorganism is more dense than its environment and nevertheless moves upward, the work done at constant speed during upward swimming exceeds that during downward swimming. The resistance hypothesis assumes that *Paramecium* can detect the direction of maximal energy consumption. The validity of this model was challenged by Kanda (1914), who reversed the density gradient applying solutions of gum Arabic; the paramecia swam upward in spite of the positive (= upward) buoyancy. Taneda (1987) used heavy water (D_2O; 1.1 g/cm^3) - avoiding the complications introduced by raised viscosity - and confirmed Kanda's observations. The physical assumptions of the resistance hypothesis agree with those of the statocyst hypothesis (see 4.3) so that conflicting evidence applies to both models. In ciliated echinoderm pluteus larvae sedimentation is completely neutralized by a compensating change in swimming velocity. This suggests the possibility that the swimming speed was sensed as a signal so as to maintain a constant velocity in any direction in space (Mogami et al. 1988). This interesting extension of the resistance hypothesis has also been considered for vertical speed regulation in *Paramecium*, but in these cells the compensation improved with the artificially raised gravitational load, which is difficult to reconcile with water-current sensing (rheoreception) (Ooya et al. 1992; see 4.8).

4.5 HYDRODYNAMIC HYPOTHESIS (ROBERTS 1970)

Due to the small size of ciliates, their swimming is primarily determined by viscous drag, whereas the roles of mass and inertia are insignificant (Reynolds number near 10^{-2}). At constant volume the slender shape of a passively driven (e.g., immobilized sedimenting) *Paramecium* (length : widths = 5) very little affects drag (+5% as compared to a sphere; Happel and Brenner 1973). The contribution of shape to propulsion in ciliates relates to cilia: a 200 μm *Paramecium* swims about 40% faster than a spherical ciliated body of the same surface area (∅ 100 μm; Keller and Wu 1977) but, eventually, size rather than streamlining determines velocity (Roberts 1981). Aluminum cell models immersed in glycerol (Reynolds number near 10^{-3}) sedimented faster with rising size (2 to 10 mm) the square root of velocity

being proportional to size of the model (Roberts 1970). This size-velocity relationship predicts that the wider (rear) half of *Paramecium* settles faster than the narrow (anterior) half of the cell. A cell in arbitrary orientation develops a gravity-induced hydrodynamic torque until the narrow anterior half is pointing upward (Roberts 1970). The practical relevance of the elegant hydrodynamic model is difficult to predict. It was objected that not all negatively gravitactic protozoa have a posteriorly shifted "centre of surface" (Winet and Jahn 1974). Grębecki and Nowakowska (1977) noted that the rate of upward reorienation of *Paramecium* was not independent of swim speed as predicted by the hydrodynamic hypothesis. Taneda et al. (1987) found that the posterior cross-section of *Paramecium* positively correlates with culture age. In a 4 days old culture, about two thirds of the cell total had posterior cross-sections exceeding in area the anterior cross-sections. The same relationship of areas of cross-sections (and, indirectly, of cell shape) applied to cells collected from the upper and lower halves of a water column. The conclusion was that hydrodynamics are no limiting parameter in gravitaxis of *Paramecium*.

4.6 PROPULSION HYPOTHESIS (WINET AND JAHN 1974)

It might be argued that because active swimming rates exceed sedimentation by an order of magnitude in *Paramecium*, cell propulsion should take the lead in graviorientation. Winet and Jahn (1974) assume that the "centre of effort" of a ciliate is anterior to the centre of gravity. This idea involves the same consequences as the static hypothesis: a more posterior location of the centre of gravity and hence negative gravitaxis (see 4.1). In agreement with the propulsion hypothesis, the analysis of metachronism in *Paramecium* suggests a minor preponderance in propulsive force of the more anteriorly located cilia, where the wave angle (as referred to the posterior direction), and implicitly the angle of the power stroke of the cilia, was slightly reduced (Machemer 1972 a, b). Winet and Jahn (1974) deduced unequal angular rates of downward and upward reorientations from the assumed interaction of gravitational and propulsive forces in *Paramecium*, in particular in cells swimming with minimal translational velocity. This was not confirmed by Nowakowska and Grębecki (1977) suggesting an alternative mechanism for negative gravitaxis.

4.7 LIFTING FORCE HYPOTHESIS (NOWAKOWSKA AND GRĘBECKI 1977)

Vectors of sedimentation and active propulsion by cilia induce forward swim ming cells to maintain a small "upward" angle between cell axis and direction of translation (see Fig. 9). This upward tilt of the leading end of the cell with respect to any direction of locomotion applies to backward swimming as well as to forward swimming, as was noted by Nowakowska and Grębecki (1977). These authors hypothesized that, analogous to a moving airfoil, relative upward tilting of the cell might generate a lifting force. If such a force would act anterior to the centre of gravity of *Paramecium*, it could generate a torque which accounts for the observed negative gravitaxis. It has been objected that shape-dependent generation of lifting forces may not apply to an actively driven body at low Reynolds numbers (Bean 1984; see 4.5). The hypothesis can be experimentally tested correlating swimming velocities and rates of upward reorientation in *Paramecium*.

4.8 SPECIAL STATOCYST HYPOTHESIS (ELECTRPHYSIOLOGICAL MODEL; MACHEMER ET AL. 1991, BABA 1991)

Gravireception in the unicellular organism differs radically from the multicellular statocyst, where the topological coordinates of a mechanically deformed sensory cell are encoded in spikes of an associated axon. For a single cell to work as a statocyst, local gravitransduction must be processed so as to induce a modulation of beating of thousands of independent cilia. Studies of the electrophysiology in ciliates, in particular of mechanoreception and ciliary electromotor coupling, indicate a principal mechanism of how gravistimulation can work in the surface membrane (Machemer and Deitmer 1985, Machemer 1988b): Antagonizing mechanoreceptor channels have gradient-type distributions in the soma[3] membrane accumulating at the polar ends of the cell (anterior: depolarizing, posterior: hyperpolarizing). Because minor depolarizations depress, and hyperpolarizations speed up ciliary activity in *Paramecium* and other ciliates (see Machemer 1986), downward swimmers reduce, and upward swimmers increase their locomotion rates. Hence, sedimentation

meets active speed regulation (gravikinesis; Machemer 1989b, Machemer et al. 1991). This membrane model of graviresponse can also explain gravitaxis using an orientation mechanism derived from helical motion (Crenshaw 1989) because decreasing negativity of the membrane potential raises the angle of ciliary beating with respect to the posterior direction. As hypothesized by Baba (1991) and Ooya et al. (1992), the increase in helical pitch angle of swimming cells causes downward swimmers to continuously turn away from the downward course, whereas an upward swimming direction is stabilized due to the decrease in pitch angle (see 8.1).

The involvement of classical sensorimotor coupling principles in graviresponses is less obvious in those ciliates, which employ statocystoids as presumed gravireceptors. Structural evidence suggests, however, that in *Loxodes* the observed connection of the statolith to the base of a modified cilium mediates gravity-induced activation of mechanically sensitive membrane channels (Fenchel and Finlay 1986a).

4.9 CONCLUSIONS REGARDING VARIOUS HYPOTHESES ON GRAVIRESPONSES

Among the published concepts of the origin of cellular graviresponses, some rely on purely physical principles (buoyancy, 4.1; hydrostatic pressure, 4.2; hydrodynamics, 4.5; airfoil-type lift, 4.7), others invoke gravireception (statocyst model, 4.3, 4.8) or rheoreception (resistance model, 4.4). So far, the hydrostatic hypothesis and rheoreception are unlikely explanations of graviresponses. Buoyancy with anterior end up (by posteriad shifts of the centre of gravity or anteriad displacement of the centre of propulsion), and hydrodynamic torque of prolate cell bodies are supported by experimental evidence. Physical and physiological models are mutually non-exclusive. The statocyst model, in its association with current electrophysiology, predicts speed regulation (gravikinesis) and reorientation by gravity. In the past, research on ciliate graviresponses was mainly focused on the orientational response (gravitaxis). Speed regulation was often ignored because it is more difficult to identify, and measurements of active propulsion are masked by sedimentation. Although gravireception does not necessarily generate a kinesis, unequivocal identification of

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[3] The term of "soma" designates the cell body (of e.g. ciliates, neurons) as distinguished from projections from that body such as cilia, flagella, suctorian tentacles, dendrites, axons, etc.

this behaviour can serve as a first step of physiological analysis of gravireception. The following chapters give a critical introduction into an electrophysiological paradigm of graviresponses in ciliates.

5. CILIATES AS EXCITABLE CELLS

The membrane potential (V_m) in a ciliate, from freshwater or marine habitat, is determined by two major electrochemical batteries, E_{Ca} and E_K, and the conductances of these batteries (g_{Ca}, g_K), as represented by concentration gradients of cations across open channels. In freshwater, the inward Ca^{2+} concentration gradient spans four orders of magnitude, so that E_{Ca} is more positive than +100 mV. With a typical outward K^+ concentration gradient of 40:1, E_K is close to -100 mV. With a conductance ratio of the resting membrane, $g_{Ca}/g_K = 1/2.5$, a resting potential near -30 mV results from ohmic voltage division (Fig. 1). The soma membrane includes, in addition to channels for homeostasis, receptor channels, which are specialized for activation by a particular stimulus modality and generate receptor potentials. These receptor potentials are graded with the stimulus strength and may be depolarizing or hyperpolarizing depending on the selectivity of the receptor channel for an ion species. So far, Ca-receptor channels (depolarizing) and K-receptor channels (hyperpolarizing) have been identified in the membrane of the cell soma (see Machemer and Deitmer 1985). According to favourable cable properties of the ciliate cell, graded receptor potentials spread rapidly with little loss. They summate and are hence the basis of stimulus integration. A special class of membrane Ca-channels, which are depolarization-sensitive, are located in ciliary membranes. Depolarization-sensitivity of these channels drives a regenerative cycle of excitation, called the Ca action potential, which accompanies rapid fluxes of Ca^{2+} into the ciliary spaces (see Machemer 1988a, Machemer and Teunis 1993). For the understanding of graviresponses, only non-regenerative excitations of the membrane by mechanically sensitive somatic channels are meaningful.

6. MECHANORECEPTION IN CILIATES

Stimulation by brief mechanical pulses applied to predetermined points of the cell membrane in *Paramecium* and *Stylonychia* has revealed a depolarizing mechanosensitivity in the anterior half of the cell; the same pulses induced hyperpolarizing mechanorecep-

tor potentials posteriad to the cytostome. Analysis under voltage clamp established two opposing spatial gradients of mechanosensitivity which overlap. The depolarizing Ca mechanoreceptor channels prevailed at the anterior cell end, and the hyperpolarizing K mechanoreceptor channels determined the mechanoresponse of the posterior cell end (Fig. 2). The data indicate that for each cell latitude a particular mechanically elicited conductance ratio, $\Delta g_{Ca}/\Delta g_K$, determines the polarity and size of the receptor potential. The conductance ratio near the cytostome was such as to suppress a receptor potential in *Stylonychia* as well as in *Paramecium* (for review see Machemer and Deitmer 1985).

Application of single mechanical pulses to the ciliate membrane revealed a comparatively low sensitivity to phasic stimulation (typical amplitudes: 5 - 10 µm; typical rise time: 5 ms). Receptor currents elicited by these pulse-stimuli involve a latency, i.e. time interval between start of membrane deformation and onset of current, of about 3 ms, which is an unusually long delay as compared to mechanoreceptors in the insect (0.1 ms) and the vertebrate inner ear (0.015 ms; Thurm 1982). Moreover, the mechanoreceptor current in *Paramecium* rises sigmoidally suggesting more than a single step of activation. These properties of the ciliate mechanoreceptor suggest that other than brief local stimuli (tonic nonlocal stimuli?) may be a more adequate mode of receptor channel activation. A daring speculation is that the pattern of mechanoreceptor conductances in ciliates reflects, in the first line, sensitivity to stimulation by gravity (Fig. 2). This so far unsubstantiated view resolves the current paradox of hyperpolarizing mechanosensitivity of the posterior cell end, where the receptor current arises synchronously with the delayed outward relaxation of the previously inward deformed membrane (Machemer and Machemer-Röhnisch 1984). Without a perspective including gravireception, posterior mechanosensitivity appears to be of little use in a predominantly forward swimming ciliate.

7. ELECTROMOTOR COUPLING

Changes in membrane potential in ciliates control the locomotor behaviour generated by modulation of the rate and direction of ciliary beating. In *Paramecium*, a minor depolarization of the membrane depresses the rate of beating at the resting potential and shifts the beat orientation in the counterclockwise direction. These combined ciliary responses lead to a reduction in the forward swimming velocity. Larger depolarizations tend
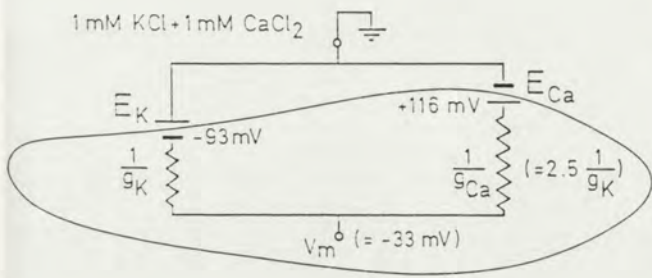
Fig. 1. Simplified electric circuit diagram of a ciliate to illustrate the determination of the membrane resting potential. The potassium and calcium electrochemical equilibrium potentials (E_K, E_{Ca}) and conductances (g_K, g_{Ca}) of the membrane channels establish the resting potential or any other potential during membrane steady-state ($dV_m/dt = 0$). V_m shifts to more depolarized potentials with Ca-channel activation ($\uparrow g_{Ca}$), and to more hyperpolarized potentials with K-channel activation ($\uparrow g_K$)
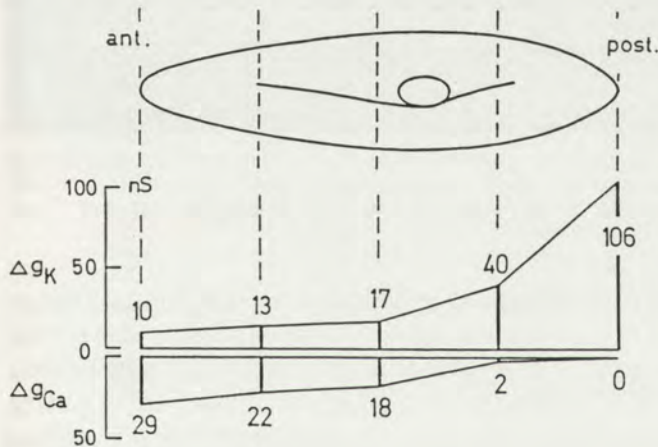
Fig. 2. Topography of mechanoreceptor channel conductances in *Paramecium* activated by local application of identical mechanical pulses to the soma membrane. In the anterior cell end (ant.), the conductance of depolarizing receptor Ca channels (Δg_{Ca}) prevails over the conductance of hyperpolarizing receptor K channels (Δg_K). In the posterior cell end (post.), only receptor K channels are activated. Stimulation at the latitude of the cytostome elicited no receptor potential because the extra conductances did not change the conductance ratio of the resting membrane (Fig. 1). Assuming that Ca and K channels for gravireception have similar distributions, gravitational pressure to the posterior cell in an upward swimming *Paramecium* hyperpolarizes the membrane; a downward swimming cell is depolarized. For a horizontally swimming cell the summed mechanoreceptor conductances from all sites (or fractions from the sums) and the conductances near the cytostome give the same ratio ($\Delta g_{Ca}/\Delta g_K = 2.3$). Local stimulation at the latitude of the cytostome gives no mechanoreceptor responses in *Paramecium* and *Stylonychia* (see Machemer and Deitmer 1985)

to inactivate the cilia or, upon regenerative amplification, to reorient counterclockwise the beat direction by about 150° ("reversal in beat orientation") together with augmentation of the beating rate; the result is a backward swimming of the cell. Hyperpolarization also augments ciliary frequency, but the beat direction turns clockwise;

such responses of the cilia raise the forward swimming rate. The complex potential-coupled motor responses of the cilia are illustrated in Fig. 3. Experiments performed in living cells and nucleotide-reactivated models of *Paramecium* and other ciliates have identified the crucial role of Ca^{2+} as a rapid intraciliary messenger during depolarization-induced ciliary activity (DCA) and hyperpolarization-induced ciliary activity (HCA) (Nakaoka et al. 1984, Mogami et al. 1990, Matsuoka et al. 1991, Mogami and Machemer 1991).
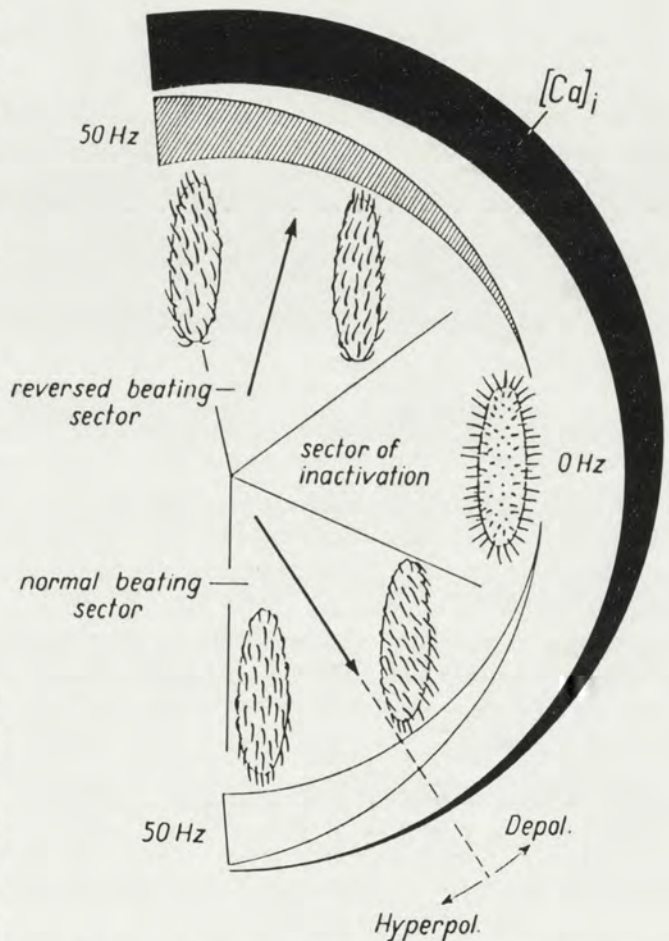

Fig. 3. Sectorial scheme to illustrate basic correlations between membrane potential, ciliary beat orientation (as seen looking down on *Paramecium* cell, anterior end up), beat frequency (curved gradients; shaded: during reversed beating), and intraciliary Ca^{2+} concentration (black gradient). In the resting state, the cilia beat toward the posterior-and-right at low frequency. Hyperpolarization raises the frequency and turns the direction of beating more posteriorly, that is, more clockwise. Any depolarization turns the beating in the counterclockwise direction. Weak depolarization depresses the beating rate with the power stroke directed more toward the right side; the cilia may fully inactivate. With larger depolarization the beat orientation is toward the anterior-and-right at more than normal beating rate. Extreme depolarization may lead to peak frequencies and an orientation slightly toward the anterior-and-left. Amounts of axonemally bound Ca^{2+} are maximal during depolarization, and minimal during hyperpolarization. Modified after Machemer (1974)
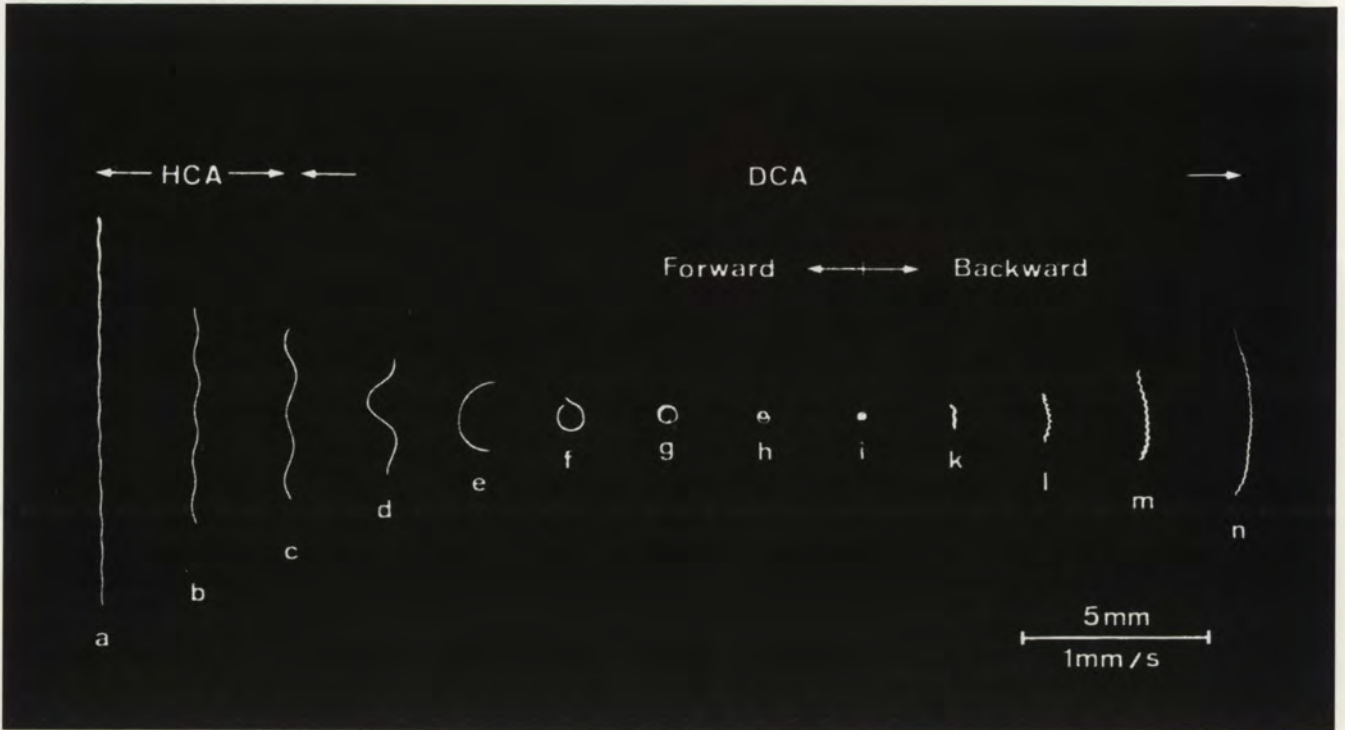
Fig. 4. Patterns of forward swimming (a - h) and backward swimming (k - n) of *Paramecium* following chemically induced hyperpolarization (HCA = *h*yperpolarization-induced *c*iliary *a*ctivation) and depolarization (DCA = *d*epolarization-induced *c*iliary *a*ctivation). Membrane negativity decreases from left to right. Swimming tracks were recorded in darkfield illumination with 5 s of photographic exposure. Track length gives the swimming velocity. Modified after Machemer and Sugino (1989)

One might expect that the coupling of ciliary responses to the membrane potential appears in the resulting swimming behaviour. This is the case (Fig. 4) as long as cells have not adapted to a lasting stimulus (Machemer 1989 a). A fully equilibrated *Paramecium caudatum* with the membrane potential at rest swims forward in a leftwinding helix at a rate at or below 1 mm/s (including about 2 to 2.5 turns; Fig. 4c).

There is agreement that helical locomotion can cancel instantaneous asymmetries in propulsive forces of the cilia providing straight net displacements of the cell (Crenshaw 1990). With gradual depolarization, the velocity of swimming decreases and the helix expands laterally (Fig. 4d, compare with Fig. 3). An even larger depolarization has sufficiently suppressed forward translation, and has widened up the helix, so that the cell appears to travel along circular arcs (as seen parallel to the axis of the helix; Fig. 4e). Further depolarization leads to rising ciliary inactivity; a weak common vector of lateral beating is thought to produce the increasingly stationary spinning of the cell Fig. 4f-i). Beyond a critical depolarization the cilia beat anteriorly driving the cell back-
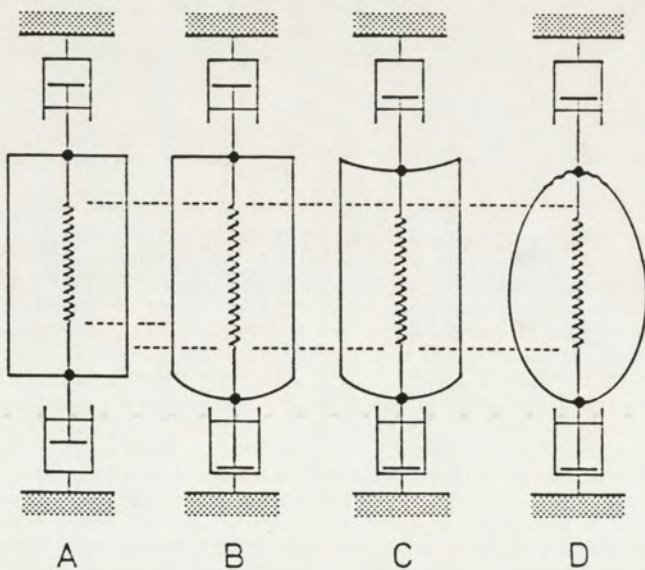
Fig. 5. Thought models of cell deformation under gravity (A to C: vertically oriented rigid cylinder with soft top and bottom; D: ellipsoid body covered by soft membrane; after Mogami and Machemer unpublished). The models are filled with visco-elastic matter and suspended in fluid with lower density. **A.** A spring connecting the upper and lower membrane represents the elastic properties; two dashpots in series represent the viscous drag of the cytoplasm. **B.** Cytoplasmic density expands the lower membrane charging the spring. **C.** Elastic pull of the spring deforms the upper membrane after some delay. **D.** With similar density and viscoelastic properties, the soft ellipsoidal model builds up outward deformation and tangential tension at the low end, and tends to release outward deformation and tangential tension at the high end. The local presentation time of gravity may determine applicability of model in (B) or in (C, D)
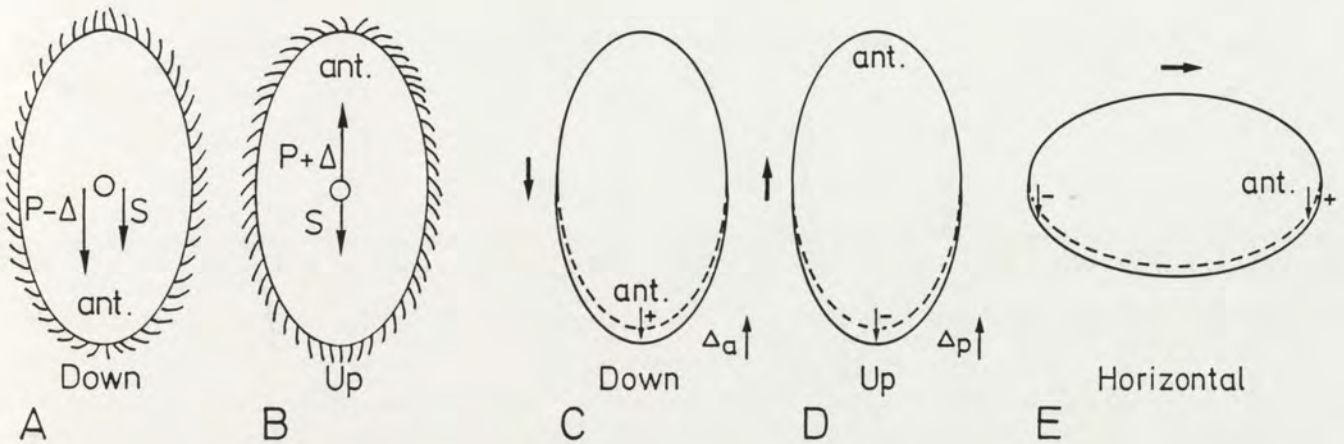
Fig. 6. Electrophysiological model of gravikinesis in a swimming ciliate (ant., anterior end of cell). **A, B**. Velocity components acting to produce an observed downward swimming (A) and upward swimming rate (B). The invariant, gravity-independent propulsion rate (P) and a gravity-induced change in propulsion (Δ) are active components; the sedimentation rate (S) is a passive component of locomotion. Velocities are proportional to acting forces according to Stoke's law. **C, D, E**. Gravity-induced outward deformation of the lower membrane (arrows), resulting shift in membrane potential (sign near arrows), and gravikinetic change (Δ) in propulsion vector (heavy arrow). **C**. Deformation of the anterior end in the downward swimming cell activates depolarizing receptor channels leading to a reduction in the resulting active propulsion (decrement: Δ_s; index "a" for "anteriorly induced"). **D**. The posterior membrane segment is deformed in the upward swimming cell activating hyperpolarizing receptor channels and raising forward propulsion (increment: Δ_p; index "p" for "posteriorly induced"). **E**. Reduced membrane deformation in the horizontally swimming cell affects both depolarizing and hyperpolarizing receptor channels, which largely neutralizes a gravireceptor response (see Fig. 2). The horizontal velocity therefore corresponds to or approximates the P-value (gravity-free propulsion rate. Modified after Machemer et al. (1991)

wards. The depolarization-dependent angle of the power stroke with respect to the cell axis and the associated beating rate (Fig. 3) determine the observed pattern of backward swimming (Fig. 4k-n).

Figure 4 illustrates that both weak depolarizations (c-f) and hyperpolarizations (c-a) modulate the pattern of forward swimming. It will be shown that changes in the net swimming rate and in pitch of the helical pathway are presumably crucial elements of physiologically guided gravikinesis and gravitaxis in ciliates.

8. PHYSIOLOGY OF GRAVISTIMULATION AND RESPONSE

The electrophysiological interpretation of the statocyst hypothesis combines existing gravitational pressure differences across the actual "lower" membrane of ciliates with well-established data of ciliate mechanoreception and ciliary electromotor coupling. From this, the behavioural consequences of stimulation by gravity are predictable in detail for experimental testing. The following paragraphs present the electrophysiological model of graviresponses and review the so far available experimental evidence in the light of this paradigm.

8.1 BASIC MECHANISMS

8.1.1 Gravikinesis

The cytoplasm is a viscoelastic body, the density of which exceeds that of the surrounding fluid. Assuming that the membrane, or structural elements associated with that membrane, undergo deformation due to the gravity-induced pressure gradient across the membrane, the actual "lower" and the actual "upper" membrane are candidates for transduction. The heavy cell body would tend to deform the lower membrane in the outward direction, and/or the upper membrane in the inward direction. Figure 5 schematically illustrates these mechanical properties of a ciliate cell assuming a vertically oriented rigid cylinder with soft top and bottom. Visco-elastic properties of the cytoplasm are thought to be represented by a spring and two dashpots connected in series to the upper and lower "membranes" (A). The heavy cytoplasm bulges the lower membrane outward charging the elastic spring (B), which deforms the upper membrane after some delay (C). In this cylindrical model, the presentation time of the gravity stimulus may be important for application of case B or C. A more realistic model of viscoelastic deformation of a cell would be an unboiled egg with the hard shell removed
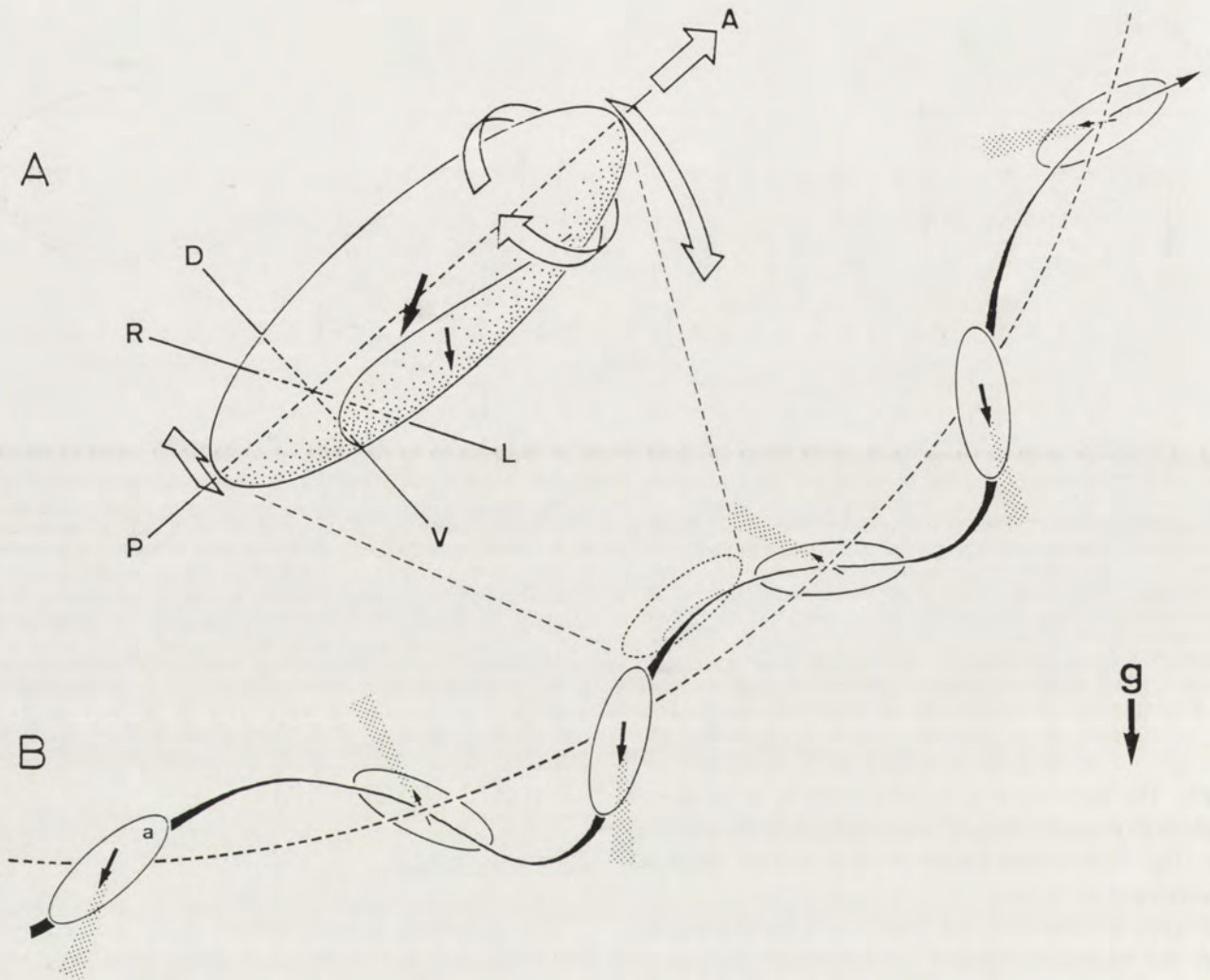
Fig. 7. Electrophysiological model of <u>gravitaxis</u> in *Paramecium* (after Baba 1991). **A.** At constant ciliary activity one translational and two rotational forces (open arrows) from summed ciliary power strokes (black arrows) combine to generate helical swimming courses about a straight axis (Crenshaw, 1990). Translation along anteroposterior axis (A-P) and lefthanded rotation about A-P axis result from the oblique direction of the power strokes (to the posterior and right; black arrows). Rotation about the right-left (R-L) axis is due to asymmetry in translational forces generated dorsally (D) and ventrally (V, in the oral groove). **B.** The gravity vector (g) acts to continuously reorient the helical swimming course upwards for the following reasons: When the anterior end of *Paramecium* (a) is turning upwards, posterior gravistimulation (Fig. 6D) reorients the ciliary power stroke (Fig. 3) more posteriorly (shaded angle) thereby decreasing the pitch and diameter of the helix, which bends its axis (dashed) upward. In the following downward-swimming phase of the cell anterior gravireceptors are activated (Fig. 6A), which induces the cilia to beat more laterally (Fig. 3), increases the pitch and diameter of the helix and further reorients its axis upward. This continuing correction of the axis repeats with every turn of the helix and leads eventually to vertical upward swimming (negative gravitaxis). With vertical orientation of the helix, local interactions of gravity with the cell do not change. Note that the oral groove of a swimming *Paramecium* faces the axis. The (not-to-scale) illustration of the track assumes that the cell proceeds along the outside of the 3D-helix; the ciliary beat orientation is here given for the dorsal side only (A: beat orientations for right and ventral sides). Original; large inset adapted after Naitoh and Sugino (1984)

and suspended in water; here, tangential tension and vertical pull are supposed to decrease in the upper membrane and to increase in the lower membrane (D).

Figure 6 explains the most straightforward assumption: deformation of, and gravitransduction in, the lower membrane. From the mechanosensory electromotor coupling properties in ciliates, this option of the hypothesis predicts a slowing of downward swimming cells and speeding up of upward swimming cells, hence a gravikinesis with negative sign as compared to sedimentation (negative gravikinesis). The corresponding velocity equations are:

$$V_D = P + S - \Delta_D; \quad V_U = P - S + \Delta_U. \quad (1)$$

With the arithmetic mean of Δ_D and $\Delta_{U \text{ termed}}$ Δ, the following simple equation results:

$$(V_D - V_U)/2 = S + \Delta. \qquad (2)$$

The second option, transduction following inward deformation of upper membranes by gravity predicts a positive gravikinesis, that is, faster active downward swimming and attenuation of active upward swimming. A third option, both upper and lower membranes transduce gravity simultaneously, leads to (virtual) neutralization of a gravikinetic response. Experimental tests of these thought-models of gravikinesis in *Paramecium* have so far strongly supported the "outward-deformation-of-the-lower-membrane   model" (Machemer et al. 1991), although the alternatives cannot be ruled out altogether.

8.1.2 Gravitaxis

It was discovered by Crenshaw (1989, 1990) that the overall direction of the helical swimming path of a microorganism changes with the pitch of the helix. Changes in pitch angle of the swimming helix (Fig. 4) are due to potential-dependent reorientations in ciliary beat direction (Fig. 3). If gravity can induce depolarizations and hyperpolarizations, effects on the form of the swimming helix and eventually on cell orientation are expected. Baba (1991) has discussed this electrophysiologically guided graded reorientation predicting a negative gravitaxis for *Paramecium*. Figure 7 illustrates gravity-induced reorientations (gravitaxis) as deduced from the electrophysiological model: Helical swimming arises from (1) ciliary beat orientation (A, black arrow) and (2) a dorsoventral asymmetry in ciliary activity (small black arrow on ventral side (V) indicating a reduced propulsive force parallel to the anteroposterior axis, A-P, of the cell). The oblique posteriad beat direction of the cilia generates the translational propulsive force (along A-P) and a lefthanded rotation of the cell (about A-P axis). The cell rotates, in addition, about the right-left axis (R-L) due to the dorsoventral imbalance of translational propulsion. The combination of one translational and two rotational movements of the cell generates a helical swimming track revolving about a straight axis with constant ciliary activity. During this swimming, the oral groove of *Paramecium* permanently faces the axis of the helix.
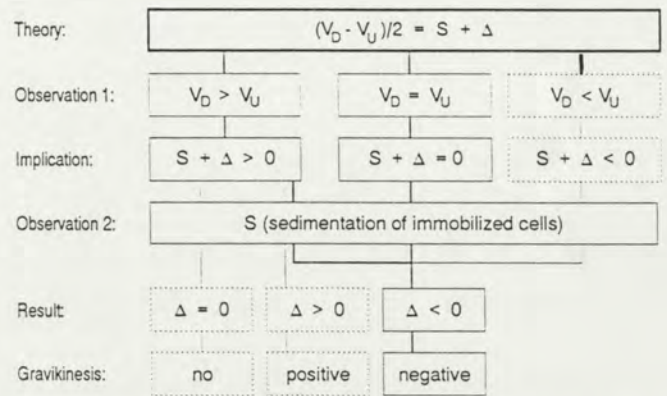
Fig. 8. Flow diagram of isolation of gravikinesis from observed vertical locomotion in a microorganism. Top box: The difference between the downward velocity (V_D) and upward velocity (V_U), divided by 2, equals the sum of sedimentation rate (S) and gravikinetic response (Δ; see equ. 2) (Machemer 1989b). Second row: Three alternative observations imply different sums of sedimentation and kinesis (0, =0, ; third row). The experimental determination of the sedimention rate (fourth row) gives the value and sign of Δ (positive sign: adding to sedimentation rate; negative sign: subtracting from sedimentation rate). Flow lines: light, possible events; heavy, observations (Machemer et al. 1991, Bräucker et al. 1992). Modified after Machemer-Röhnisch et al. 1993)

Gravity is transduced in the posterior cell membrane during upward oriented sections of swimming (B; a = anterior end). The posteriad reorientation of ciliary beating decreases the pitch and diameter of the helix; hence the axis of helix (dashed) bends upward. When the cell enters a downward-oriented part of the track, gravistimulation near the anterior end induces a more lateral reorientation of all cilia, which increases the pitch and diameter of the helix leading to further upward bending of the axis of the helix. The interaction of the gravity vector with helical swimming generates a sequence of clockwise and counterclockwise reorientations of the cilia and resulting adjustments of the swim path, until the axis of the helix is in the vertical. Here, orientation stabilizes because the gravity vector acts on the same membrane area at any time of advancement along the helix.

8.2 IMPLICATIONS OF THE ELECTRO-PHYSIOLOGICAL MODEL

8.2.1 Masking of the Graviresponse

In all cells so far tested, the gravity-induced active and passive responses overlap. An observer may note, for instance, that the downward velocity exceeds the
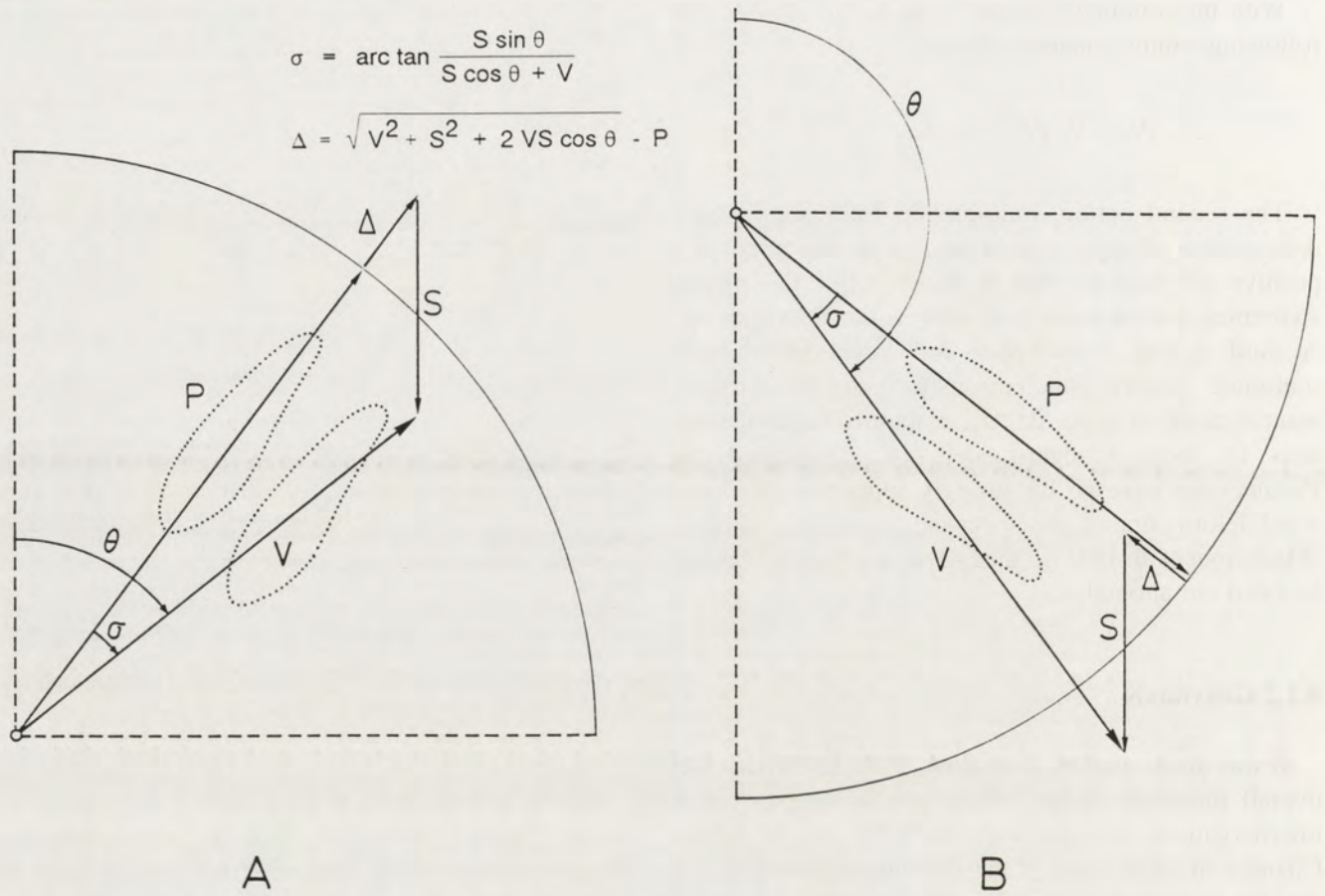
$$\sigma = \text{arc tan}\ \frac{S \sin \theta}{S \cos \theta + V}$$

$$\Delta = \sqrt{V^2 + S^2 + 2 VS \cos \theta}\ - P$$

A

B

Fig. 9. Relationships between the velocity vectors of active propulsion (P, Δ), sedimentation (S) and the resultant (V) in the vertical plane as functions of inclination angle (θ). The scheme assumes that the gravikinetic component of propulsion (Δ) antagonizes S (equ. 1, 2). **A.** Upward swimming cell (dotted contour; θ < 90°). Sedimentation induces the course (P) to settle by angle σ, now proceeding along V. The same angle (σ) occurs between the major cell axis and V. **B.** Downward swimming cell (θ > 90°). As during upward swimming, the axis of the cell is off the course of V by σ. The value of σ is calculated from the observed values of V, S and θ. For any direction in space, Δ results from V, S, θ and P. In *Paramecium*, P corresponds to the horizontal velocity (see Fig. 18)

upward velocity, but this is, regarding graviresponses, equivocal information (Fig. 8). Only with measurements of downward and upward velocities, and of the sedimentation rate, it is possible to know the sign and size of a gravikinetic response (Δ; positive sign: parallel to sedimentation; negative sign: antiparallel to sedimentation), or the absence of such response. By the same reasoning, an identity of observed downward and upward locomotion rates suggests a perfect neutralization of sedimentation and gravikinesis.

8.2.2 Effects of Cell Orientation

With a given angle of inclination (θ) from the direction vertical-up, the sum of the active propulsion vectors (P + Δ) and sedimentation (S) gives the resulting velocity vector (V). The directions of P and V include

an angle (σ), which is determined by S, V and θ according to the equation

$$\sigma = \text{arc tan}\ \frac{S \sin \theta}{S \cos \theta + V}\ , \tag{3}$$

and Δ results from P, S, V and θ:

$$\Delta = \sqrt{V^2 + S^2 + 2VS \cos \theta}\ - P . \tag{4}$$

Figure 9 shows why the observed velocity (V) commonly differs from the gravity-invariant velocity (P) due to vector addition of the variables Δ and S. A frequent observation (e.g., in *Paramecium*; Machemer et al. 1991) is that V is less than P in upward swimming cells (Fig. 9A), and larger than P in downward swimming cells (Fig. 9B). It is possible that the scalar values of P and V coincide at any given orientation due to
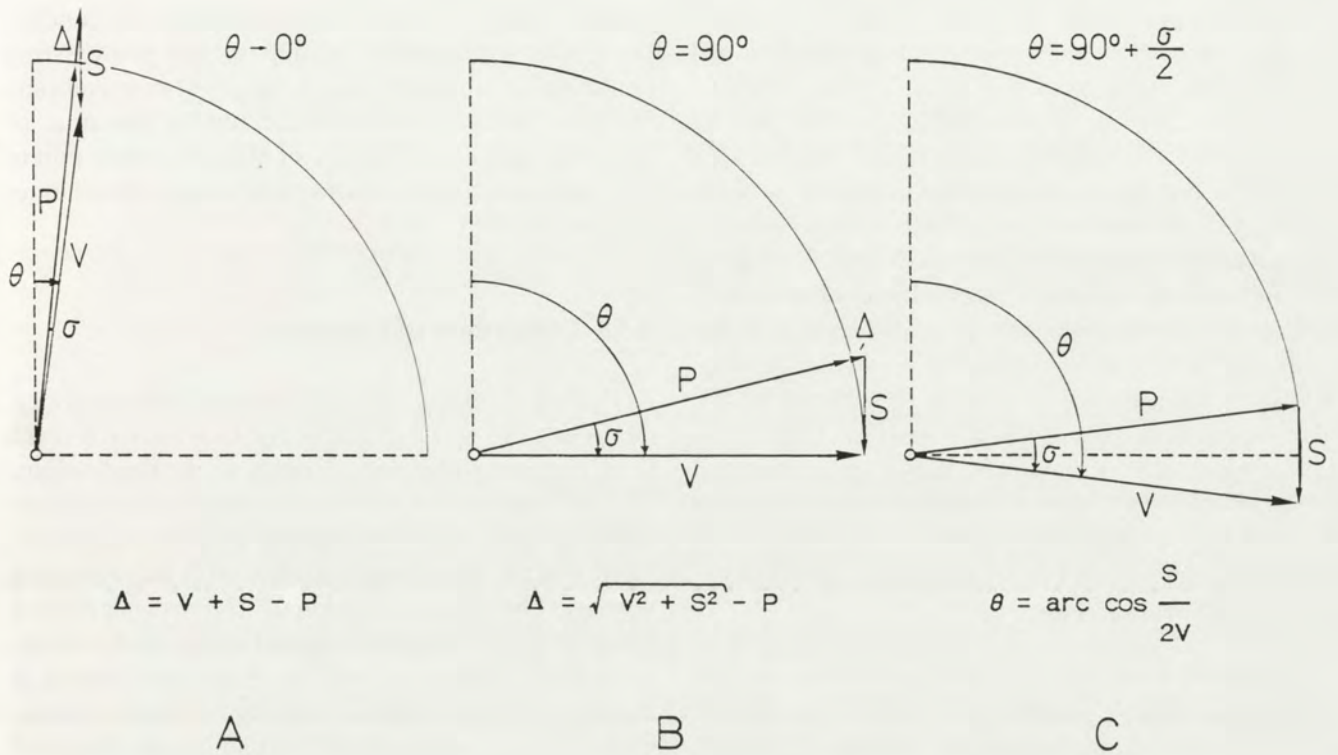
Fig. 10. Special relationships between velocity vectors and inclination angle (θ). **A.** With $\theta \to 0°$, the sum of P and Δ equals the sum of V and S. **B.** With $\theta = 90°$, the value of Δ is found applying the pythagorean law. **C.** For an inclination slightly beyond 90°, the resulting velocity (V) is equal to the invariant propulsion (P; $\Delta = 0$). This inclination angle (commonly between 90° and 96°) is calculated from S and V. See text for applications

special vector sums of Δ and S (*Loxodes*; Bräucker et al. 1992). Figure 9 also illustrates that sedimentation introduces an angle (σ) subtended between the major cell axis and the observed swimming direction.

With the angle of inclination (θ) decreasing toward 0°, velocity vectors become aligned according to the equation: P + Δ = V + S (Fig. 10A). With θ approaching 180°, P - Δ = V - S. In vertical orientations the gravikinetic velocity component directly results from three experimentally accessible parameters. In horizontally moving cells ($\theta = 90°$; Fig. 10B), the scalar values of P + Δ approximate the horizontal velocity (V_H) because Δ is small. In *Paramecium*, P = V_H (see 8.4.2); hence, a resulting velocity equal to P is expected to occur along a slightly downward slope (Fig. 10C). Because the line of viewing at horizontal chambers is vertical, those V-values of cells, which swim obliquely upward or downward, are seen reduced in size (Fig. 9). The possible minor errors encountered in assessment of horizontal velocities of cells in horizontally oriented chambers have no practical meaning.

8.3 CIRCUMSTANTIAL EXPERIMENTAL CONDITIONS

The behaviour of microorganisms is highly dependent on the conditions of steady-state and under stimulation. In ciliates, adaptation to chemically altered environments covers several hours (see Machemer 1989a). At the time of this review, the data on graviresponses in ciliates are still too limited to define those experimental conditions, which can crucially affect reproducibility of the results. Here, we briefly list some parameters.

8.3.1 Cell Cultures and Experimental Solutions

Ciliates are often grown in organic media (e.g., infusions of hay, lettuce etc.) which supply a rich bacterial fauna. The chemical composition of these media is undefined. Transfer of cells in the late logarithmic phase of reproduction to defined experimental solutions is therefore advised for data reproducibility. The state of feeding affects gravitaxis (4.1); cell size affects the rate of sedimentation (4.2).

Chemically defined inorganic solutions are well tolerated by ciliates, and some are even suited for cell reproduction. The composition of these media is nevertheless not *ad libitum*. Because ciliates use Ca^{2+} and K^+ electrochemical gradients in the first line, these cations should be present in the external solution in about millimolar concentration. Na^+ and Mg^{2+} raise the rate of intrinsically triggered action potentials and reversals in beat orientation. Elevated concentrations of K^+ and/or Na^+, and extreme reduction in Ca^{2+} depolarize the membrane in *Paramecium* inducing low swimming rates and, potentially, curved swimming (Machemer 1989a, Machemer-Röhnisch and Machemer 1989, Ooya et al. 1992), which affects gravitaxis. Tap water includes all these cations in varying concentrations and is therefore unsuited for experimental solution.

8.3.2 Equilibration Time

Changes in solutions and other environmental conditions involve adjustments of the membrane potential and membrane input resistance, which cover several hours (Oka et al. 1986). Responses to given depolarizing and hyperpolarizing stimuli depend on the stimulus-induced membrane conductance for Ca^{2+} and K^+ added to the pre-existing conductance for these ions. A steady-state membrane potential, which is still off the resting potential of the fully equilibrated cell, can affect receptor potential size and ciliary motor responses (Machemer 1989a).

8.3.3 Proportions of Chamber

Optical recording of cell behaviour commonly requires that the space available for locomotion is much limited in one dimension. A depth of the fluid volume of 1 to 2 mm and focusing to the central sheet of water has often been used for ciliates to avoid hydrodynamic "wall effects" which may interfere with the swimming velocity (Wu 1977). Limitation to ≤2 mm depth of fluid, however, is no safeguard against convection currents from local heating or cell accumulations joining in unidirectional locomotion. Rectangular lateral dimensions of the inner space for swimming are less well suited for recording graviresponses than square or circular shapes because cells tend to be preferentially
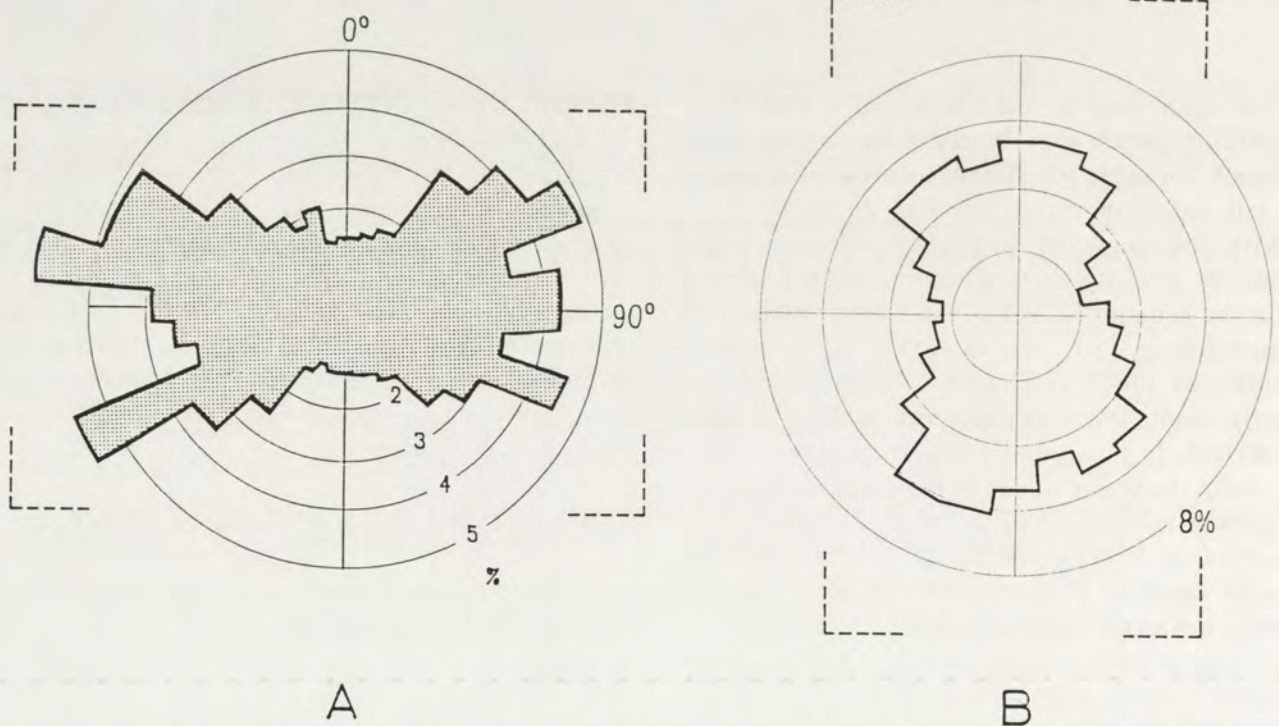
Fig. 11. Fluid-space proportions of the horizontally oriented experimental chamber (dashed marks) affect distributions in *Paramecium caudatum* (arcs: percent of total). **A.** Dimensions: 26 mm (0°), 41 mm (90°); direction coefficient: $r = 0.07$ from 899 traces (Machemer et al. 1991). **B.** Dimensions: 30 mm (0°), 18 mm (90°); direction coefficient: $r = 0.03$ from 9789 traces (Machemer et al. 1992). In both cases low direction coefficients indicate absence of sidedness of the distributions

oriented along the major axis of the fluid space for unknown reasons (Fig. 11). A disadvantage of circular ponds is that they invite swimming along the periphery (Dembowski 1924) leaving the central recording area comparatively empty.

8.3.4 Mechanical Disturbances

Ciliates are highly sensitive to omnidirectional shock, vibration, or mechanical shear as introduced during cell transfer to new solutions. Depending on the special organization of mechanosensitivity, the cells speed up (*Paramecium*) or reverse (*Stylonychia*). Tests have shown that a raised swimming rate of *Paramecium* from transfer between identical solutions declines to the normal level within 5 min (Machemer-Röhnisch and Machemer 1989). Changes in acceleration may induce different behavioural responses in *Paramecium* than the persistent gravity input (Murakami, personal communication). This suggests a careful design of experiments involving changes in acceleration to avoid overlapping of responses to two different modes of mechanical stimulation.

8.3.5 Temperature

The rates of ciliary beating and of locomotion are strongly dependent on the surrounding temperature (Machemer 1972 b, 1974) suggesting that experimental temperatures be kept constant in electrophysiological and behavioural experiments. In addition, the culturing temperatures modify the electric membrane properties and behaviour for a defined experimental temperature (Martinac and Machemer 1984, Tominaga and Naitoh 1992) so that culturing and experimental temperatures are to be considered in experiments testing gravisensitivity. In particular, temperature gradients in the experimental chamber, which are potentially introduced by illumination, can lead to receptor potentials and thermoaccumulation of the highly temperature-sensitive ciliates (Nakaoka et al. 1987, Inoue and Nakaoka 1990, Tominaga and Naitoh 1992).

8.3.6 Illumination

"Colourless" ciliates may respond to light intensity and spectral composition showing photoaccumulation (Iwatsuki and Naitoh 1982, 1983) as observed in cells including green symbionts (Reisser and Häder1984, Matsuoka and Nakaoka 1988) or pigments (see Colombetti 1990). *Loxodes* absorption and action spectra show peaks at 360 nm and 435 nm, and minimal photosensitivity at >500 nm (Finlay and Fenchel 1986), and transition from the dark to 10 klx of white light induces a positive gravitaxis in this species (Fenchel and Finlay 1986b). For elimination of photoresponses superimposed on responses to gravity, minimal light intensity, an even illumination of the field for recording, and a band width near the minimum of spectral sensitivity of the experimental cell are advised. Additional infrared filtering is a precaution. Narrow-bandwidth infrared illumination may be useful, if electric and behavioural responses from cell heating can be excluded. A careful documentation of the intensity and spectral data assist comparison between different experiments. Darkfield illumination gives optimal contrast for data evaluation.

8.3.7 Aeration

Ciliates commonly tolerate a wide range of oxygen tension, but the degree of air saturation of the culture and experimental medium may affect the graviresponse. In *Loxodes* a positive gravitaxis was observed at high tension of O_2, and a negative gravitaxis at extremely low O_2 (Fenchel and Finlay 1984). This ciliate was characterized as a microaerophile accumulating in strata of low oxygen concentration (≤5% atm. O_2; Finlay et al. 1986). The swimming velocity of *Loxodes* declines together with both O_2 tension and light intensity (Fenchel and Finlay 1986b). In *Paramecium* negative gravitaxis and swimming velocity rose with the air saturation decreasing (≤20% atm. O_2; Hemmersbach-Krause et al. 1991). However, in a previous investigation on the effects of various O_2 tensions and adaptation times on graviresponses of *Paramecium*, gravitaxis declined with adaptation, and gravikinesis correlated with swimming velocity, whereas a direct effect of O_2 on gravitaxis and gravikinesis was not established (Machemer et al. 1993). The mechanism of interference of O_2 with the graviresponses remains to be further analyzed.

8.3.8 Determination of Sedimentation Rates

The density-induced settling of ciliates cannot be determined in actively moving cells (unless the

instantaneous vertical displacement rate is deter-
mined upon step-transition from normal gravity to
microgravity). Artificial halting of active locomotion
reveals a sedimentation rate of cells with the cilia
immobile. For a long time NiCl$_2$ has been used for
ciliary immobilization (Gelei 1935). The Ni^{2+} revers-

ibly binds to 14S dynein of the ciliary axoneme
inhibiting active sliding between the microtubular
doublets (Larsen and Satir 1991). Addition of 30 µM
Ni^{2+} can fully arrest the cilia of permeabilized,
ATP-reactivated *Paramecium* (Larsen and Satir
1991), but due to slow entrance into live cells a much
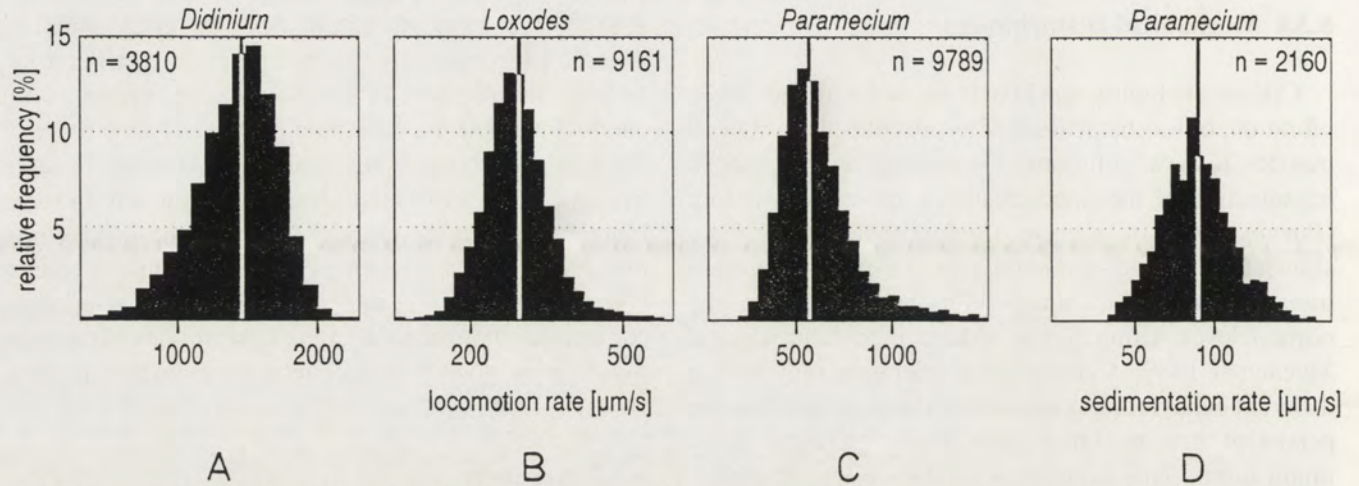
Fig. 12. Percentages of velocity-class distributions in horizontal unbiased movement of 3 species of ciliates (**A** - **C**) and in sedimenting *Paramecium* (**D**). All cells were equilibrated for 3^h at 90% atm. O$_2$ at dim light in defined experimental solution and kept at 22°C prior to recording. Velocity distributions are non-Gaussian in *Didinium* and *Paramecium*. The vertical lines give median velocities (A, 1350 µm/s; B, 293 µm/s; C, 566 µm/s; D, 89 µm/s); n = number of evaluated traces. Unpublished
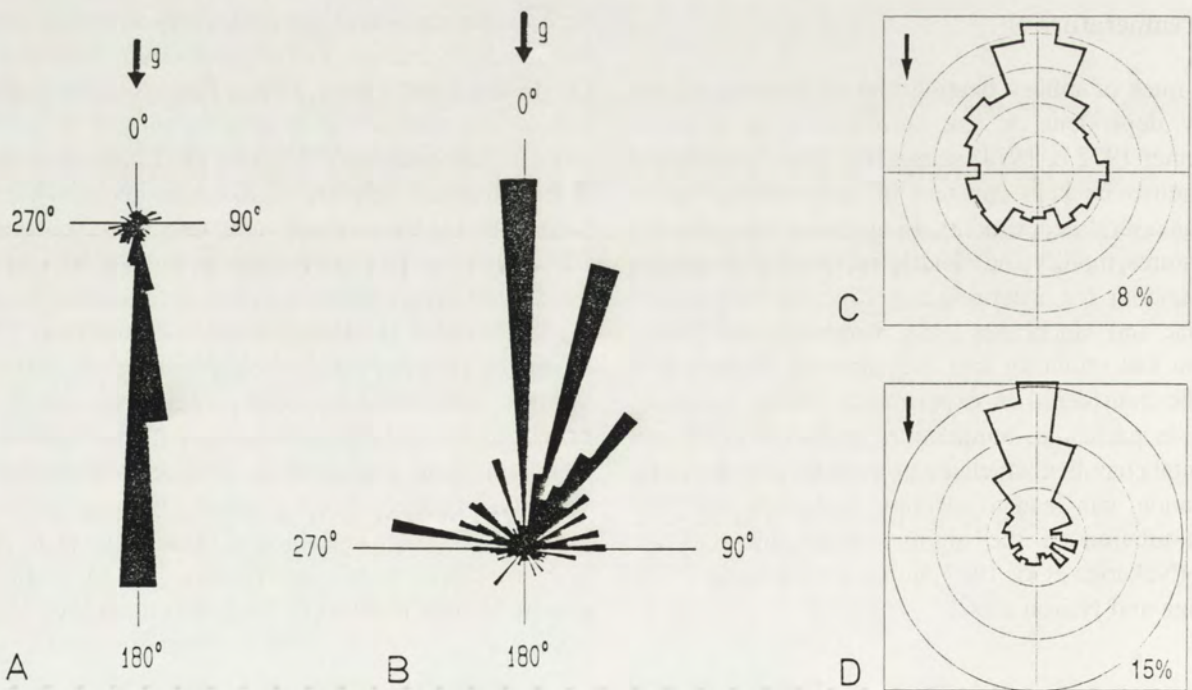
Fig. 13. Gravitactic response variations in four different protists (A, B, dinoflagellates; C, D, ciliates). Arrows indicate the direction of the g-vector (g; downward) **A.** *Peridinium faeroense*; positive gravitaxis (*r*-value 0.51). **B.** *Amphidinium caterea*; negative gravitaxis (*r*-value, 0.46). **C.** *Paramecium caudatum*; negative gravitaxis (orientation coefficient 0.200) **D.** *Didinium nasutum*; negative gravitaxis (orientation coefficient 0.488) in (C) and (D) r_o=r. Definitions of coefficients of direction (*r*) and orientation (r_o) see 8.3.10.4. A, B, Eggersdorfer and Häder (1991) with permission; C, D, Machemer et al. (1992) with permission
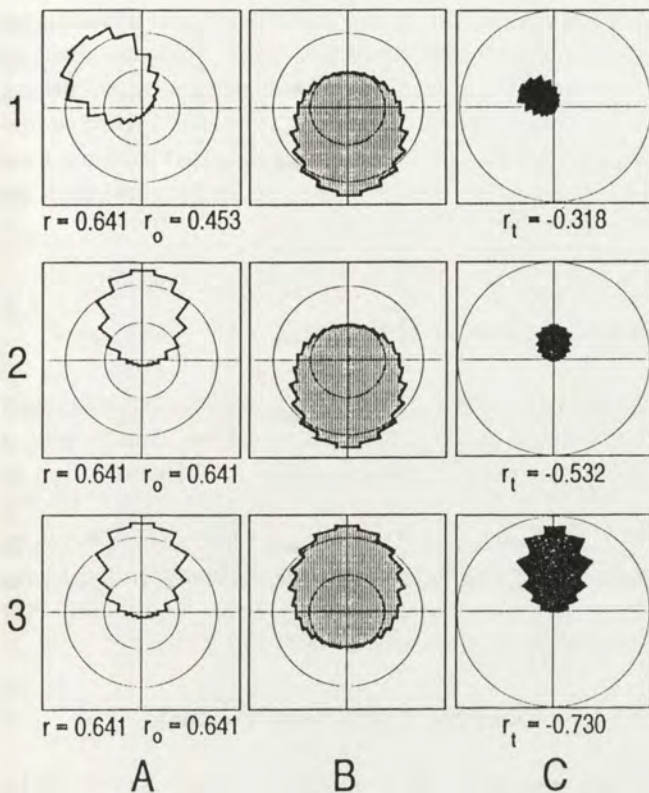
Fig. 14. Modelling three different coefficients of polar distributions (r, r_o, r_t) for characterization of gravitaxis. **A.** Circular histogram of orientation illustrating values of r and r_o. The *direction coefficient* (or r-value) gives the "centre of direction" neglecting orientational preciseness with respect to the gravity vector (A1, 2: $r = 0.641$). The *orientation coefficient* (r_o) incorporates the orientational response with respect to the gravity vector (A) multiplying the r-value with the cosine of the vector angle (A1: $r_o = 0.453$; A2: $r_o = 0.641$). **B.** Circular histogram of velocity associated with orientation in (A). **C.** Circular histogram of gravitaxis including both the orientational and velocity components and illustrating r_t as the "centre of tactic response". The *taxis coefficient* (r_t) incorporates the sign of gravitaxis (negative: upward; positive: downward) and cell distributions due to velocity (B) and orientation (A). The taxis coefficient grows (toward unity) with rising orientation (A1, 2) and/or rising velocity in the preferred orientations (B2, 3)

raised concentration (about 1 mM $NiCl_2$ in experimental solution) and about 20 min exposure are required for reliable immobilization of an experimental population (Machemer et al. 1991). With even higher concentrations of Ni^{2+} (Ooya et al. 1992: 5 mM) and longer times of exposure, the cells increasingly deteriorate; the cell bodies tend to round up, thereby increasing the sedimentation rate for hydrodynamic reasons (Happel and Brenner 1973). A remaining problem of Ni^{2+}-immobilized ciliates is that the settling velocity of immobilized cells may differ from that of actively moving cells of the same size and density.

Even the most careful immobilization by Ni^{2+} cannot fully eliminate residual ciliary activity, which may occur in a small proportion of the population and is then seen in recessed parts of the oral groove. These cells often drift away from the vertical path of sedimentation and are thereby identified. A more dangerous source of error is a clustering of groups of immobilized cells leading to artificially raised sedimentation rates and local convection. Convection currents (carrying small particles at the same speed) can be identified.

8.3.9 Acquisition of Data

Graviresponses of motile organisms have been assessed qualitatively (in which direction do cells move, where do they accumulate?) or using quantitative methods. *Individual cells* were observed either documenting the swimming course (Fukui and Asai 1985) and/or taking the time and distance covered on a vertical plane to know the direction and speed of the cell (Fenchel and Finlay 1984, Taneda, 1987). A quantitative approach using *large numbers of cells* is the determination of cell numbers at different vertical levels (Fenchel and Finlay 1984, Häder and Griebenow 1988) and calculation of mean vertical velocities (Fenchel and Finlay 1984) applying mathematical diffusion approximations (see Lapidus and Levandowsky 1981).

Contemporary equipment for videorecording has increasingly simplified quantitative documentations of cells in large populations without sacrificing individual behaviour. Two-dimensional imaging of swimming cells at regular framing rates (25 or 30 Hz) is a valuable basis of diverse evaluation methods (see Inoué 1986), establishing the velocity, direction and form of cellular locomotion. The recording of ciliate swimming tracks in three dimensions has previously been introduced using two coupled videosystems (Baba et al. 1991). This method will be particularly suited for detailed analyses of the modification of swimming helices under gravity (see 8.1.2).

Two alternative strategies exist regarding data acquisition: (1) On-line sequential sampling and evaluation of single cells, which are automatically tracked for some time period (typically 200 to 300 ms; Häder and Vogel 1991). This fully automated and efficient method uses images of cells, which are large enough for identification of their centroid. The documentation of large cell samples covers some period of time (up to 500 specimens per minute). Necessarily, the number of individual cells within the recorded sample is difficult

to estimate, and particular types of locomotion, such as reversals, cannot be separated. (2) Simultaneous recording of a field of 100 to 300 individual cells (Machemer et al. 1991, 1992; Bräucker et al. 1992). A following evaluation of superimposed videofields from a typically 2 to 4 s time period is accessible to identification, time sequence, classification and documentation of individual tracks collected during very limited time periods. This latter method, which is so far not fully automated, involves more time-consuming evaluation procedures.

8.3.10 Processing and Presentation of Data

8.3.10.1 Behavioural Variety

Strategies for evaluations of graviresponses in ciliates are now mainly directed at conclusions extracted from a representative number of (1) individual cells and/or (2) repeated readings from the same cell. The assessment of data from a large number of individual cells is important because a great variety exists between individuals in the orientational and kinetic behaviour even under most carefully controlled experimental conditions. Figure 12 gives an example of the distribution of horizontal swimming rates in *Didinium*, *Loxodes* and *Paramecium* (A-C) and the sedimentation rate in *Paramecium* (D). A common property of these distributions is that velocities vary within one order of magnitude with extremes separated by factors of 4 or more. With an observed velocity determined by at least 3 independent variables (invariant velocity, sedimentation rate, gravikinetic response; see 8.1.1), variations in individual gravikinetic responses are large. Until the contributing parameters of gravikinetic responses are fully identified, conclusions are based on generalizations from comparatively large cell numbers. The same argument applies to orientational responses to gravity. Figure 13 gives circular histograms of the gravitaxis in four species of protists, each based on evaluations of several hundred tracks. These histograms can be interpreted and compared using additional quantifying methods.

8.3.10.2 Statistical Treatment

Figure 12 shows non-Gaussian velocity distributions in three ciliate species. These distributions demonstrate that calculations of arithmetic means and standard devia-

tions would not be applicable. Nonparametric statistics (medians and confidence intervals), apply to all cases including Gaussian and non-Gaussian distributions. Careful assessments of mean velocities are most important because gravikinesis is calculated from medians of observed swimming rate, sedimentation, and invariant velocity (Machemer et al. 1991, 1992).

8.3.10.3 Circular Histograms

Graviresponses of a cell population are adequately represented using circular histograms, which plot a median velocity or percent of cell total found within an orientational sector of predefined angle (Figs. 11, 13, 17, 19). Definitions of sector angle compromise between size of cell sample and angular accuracy. In the literature, sectorial angles range between 5.625° and 90°. Frequently used sectors are 15° and 30°.

8.3.10.4 Direction Coefficient (r-value)

According to circular statistics (Batschelet 1981), polar distributions of swimming directions are characterized by a coefficient, r, which is the value of the *resultant* vector of all individual directions (β_i). The value of r is calculated from the coordinates of the "centre of directions" (x, y):

$$r = \sqrt{\bar{x}^2 + \bar{y}^2} \, ,$$

with the coordinates determined from the individual directions:

$$\bar{x} = \frac{\sum (\cos \beta_i)}{n} \, ; \qquad \bar{y} = \frac{\sum (\sin \beta_i)}{n} \, ,$$

where n is the number of data. The scalar value of *r* does not indicate the orientation of a polar distribution with respect to the stimulus source (Fig. 14A 1, 2).

8.3.10.5 Orientation Coefficient

Defining the gravity vector as parallel to the 0°-180° axis, and multiplying the *r*-value with the cosine of the resultant orientational angle (Φ) gives the orientation coefficient (r_o; Machemer et al. 1991, Fig. 14A 1, 2):

$$r_o = r \cos \Phi \, , \tag{5}$$

The orientation coefficient is +1, if all cells are strictly oriented upward (0°); $r_o = -1$, if all cells are oriented

downward (180°), and $r_o = 0$, if all cells are oriented at 90° or 270°, or the distribution is random. The angle of the mean orientational vector (Φ) is defined as follows:

$$\bar{x} > 0 : \Phi = \arctan(\bar{y}/\bar{x}) ;$$
$$\bar{x} < 0: \Phi = 180° + \arctan(\bar{y}/\bar{x}) .$$

For x = 0, special further conditions apply (Batschelet 1981).

8.3.10.6 Taxis Coefficient

An orientation coefficient may not correctly account for an observed gravitaxis, if the swimming rate was modified by gravity due to sedimentation and/or kinesis (Fig. 14B 1, 2). Preferred upward orientations (Fig. 14A 2) will be less effective on gravitaxis (C2), if the velocity of upward swimmers was reduced as compared to downward swimmers (B2), and negative gravitaxis will be more pronounced (C3), if upward swimmers were faster than downward swimmers (B3). Weighting individual swimming angles (β_i) with individual swimming rates (v_i) at these angles,

$$\bar{x} = \frac{\sum[(\cos\beta_i) v_i]}{n} ; \qquad \bar{y} = \frac{\sum[(\sin\beta_i) v_i]}{n} ,$$

the taxis coefficient, r_t , is calculated (Machemer et al. 1991):

$$r_t = \frac{-r\,n\cos\Phi}{\sum v_i} . \qquad (6)$$

The sign of the taxis coefficient corresponds to common definitions of gravitaxis. The r_t approximates the value of -1 with a perfect negative gravitaxis, that is, optimal upward orientation and upward swimming velocity; r_t is +1 with a perfect positive gravitaxis, and $r_t = 0$, if the cell population splits ("neutral gravitaxis"; Machemer-Röhnisch et al. 1993) or shows no gravitaxis at all. The numerical consequences of characterization of graviresponses by r, r_o and r_t are listed in Table 1.

8.3.10.7 Kinesis Coefficient

A taxis coefficient accounts for orientations and observed vertical velocities but not for gravikinesis, which is the increment (or decrement) in velocity after accounting for sedimentation. A measure of gravikinesis is the kinesis coefficient, r_k, which is the ratio of propulsion velocity change (Δ) over invariant velocity (P) (Machemer et al. 1991):

$$r_k = \Delta/P . \qquad (7)$$

Table 1

Diagnosis of polar distributions of cells under gravity using the direction coefficient (r), orientation coefficient (r_o) and the taxis coefficient (r_t). Model distributions of orientation and velocity (Fig. 14) are shown in different combinations to illustrate the impact on quantitative assessment of gravitaxis. Directions of summed vectors of taxis and velocity are indicated by arrows (gravity is downward). It is seen that a large variety of possible tactic responses receives identical r-values. The *direction coefficient* may represent gravitaxis (without sign), on the condition that the vector of summed orientation occurs vertically upward, and velocity is the same in all directions. The *orientation coefficient* gives the preciseness of gravitactic orientation but excludes, *per definitionem*, the velocity component of the graviresponse. The sign and size of the *taxis coefficient* most completely characterize the observed gravitactic response

Bias of Direction	Velocity	r	r_o	r_t
				-0.730
			0.641	-0.641
				-0.532
				-0.576
		0.641	0.453	-0.453
				-0.318
				-0.158
			0.000	-0.000
				+0.158

The kinesis and orientation coefficients cannot be summed to give the taxis coefficient, as seems to be the case with small kinesis coefficients in *Paramecium* (Machemer et al. 1991). In gliding *Loxodes* gravikinesis fully compensates sedimentation at 1 g (Bräucker et al. 1992) and cell orientation is at equal proportions either upward or downward (Machemer-Röhnisch et al. 1993). Here, the coefficients of orientation and gravitaxis are near zero, although the kinesis coefficient may be large.

8.4 CURRENT EXPERIMENTS

The design and analyses of experiments on graviresponses have recently changed due to the availability of new research tools, in particular short-term and intermediate-term microgravity, hypergravity, and efficient processing of large numbers of data. Here, we emphasize these new approaches. Beyond giving a brief overview (see paragraph 4), no attempt is made to fully assess the previous literature.

8.4.1 Experiments under Normal Gravity

8.4.1.1 Free Locomotion

Experiments using *Paramecium* confirmed previous observations of negative gravitaxis (see for example Fig. 13C). The orientational response was more pronounced after gassing with N_2 and following 180° turns of the cuvette (Hemmersbach et al. 1990). Either manipulation involves mechanical disturbances, which raise the swimming speed (Machemer 1988 b) and gravitactic orientation (Bozler 1926, Machemer unpublished observations). Lowering the O_2 tension of the medium improved negative graviorientation and raised the swimming velocity indicating a possible active regulation of gravitaxis (Hemmersbach et al. 1991).

Gravity-induced speed modulation was documented in *Tetrahymena* (Mogami et al. 1988) and in the dinoflagellate *Peridinium* (Häder and Liu 1990, Häder and Vogel 1991). Mogami et al. (1988) noted that in a vertical chamber *Tetrahymena* tended to swim faster upwards (0.3 mm/s) than downwards (0.2 mm/s, n = 1086; 18°C). At higher temperature (25°C), the velocity (0.37 mm/s, n = 1313) was the same in upward and downward swimmers. These data suggest active speed regulation by gravity because rates of downward swimming should exceed those of upward swimming, if gravity would act only physically causing sedimentation. Machemer et al. (1991) documented downward and upward swimming rates in *Paramecium* with the difference exceeding the value of twice the sedimentation rate. Because V_D - S and V_U + S differed significantly from the arithmetic mean of V_D and V_U, these data proved the existence of a gravikinetic component (Δ) of velocity (equ. 2). Subsequent experiments using free swimming *Paramecium* and other ciliates confirmed the existence of a gravikinetic velocity, which subtracts from the velocity of sedimentation (Machemer et al. 1992, Ooya et al. 1992, see Table 2).

8.4.1.2 Velocity-dependence of Gravikinesis?

Ooya et al. separated in a population of *Paramecium caudatum*, which had been adapted to Dryl's solution[4] for at least 1 h, slow swimmers with a *curved* path and fast swimmers pursuing a more or less *straight* path. Isolation of the gravikinetic response in these groups showed that the negative gravikinesis was more pronounced in the slower, curved swimmers (Fig. 15). While curved swimming may result from the special composition of the experimental solution, it is possible that a reduced velocity (associated with swimming in wide helices, Fig. 4e) is a favourable condition for large values of gravikinesis. Alternatively, local presentation time of the gravity stimulus, which is comparatively longer in straight as compared to curvy swimmers, may affect the size of the gravikinetic response (Fig. 5C).

8.4.1.3 Cells under Galvanotactic Alignment

The velocities of vertically swimming cells (V_D, V_U) and the sedimentation rate (S) give peak values of the gravikinetic response (equ. 2; Fig. 10A). Establishing a vertical, linear DC field across the experimental chamber is useful for obtaining large numbers of data from vertically swimming cells. A weak voltage gradient sufficient to align the cells increased horizontal swimming rates of *Paramecium* by 36% (from 834 to 1136 μm/s; Machemer et al. 1991). The term of $(V_D - V_U)/2$ used for calculation of gravikinesis (Δ; equ. 2) largely cancels galvanotactic augmentation of swimming, which allows a rough approximation of gravikinesis (-38 μm/s; Machemer et al. 1991). However, the galvanotactic increment in velocity may not be fully symmetric in downward and upward swimming cells which would introduce an error in assessment of Δ.

8.4.1.4 Swimming in Solutions of Adjusted Density

Ooya et al. (1992) have used a colloidal solution of Percoll (Sigma) in Dryl's solution raising the

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[4] Dryl's solution is characterized by a high content of Na^+ and the lack of K^+: 2 mM Na-citrate + 1.2 mM Na_2HPO_4 + 1.0 mM NaH_2PO_4 + 1.5 mM $CaCl_2$ (Dryl, 1959).
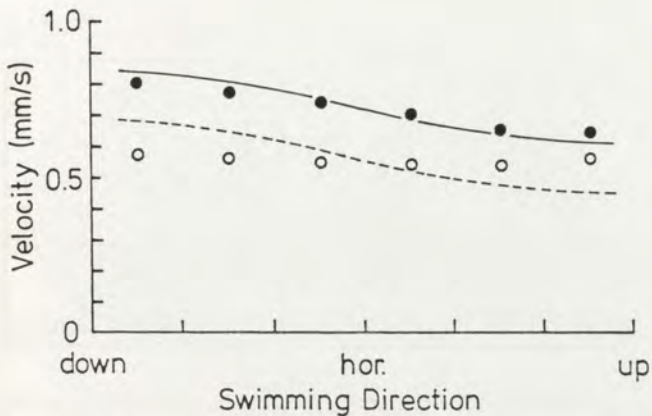
Fig. 15. Average swimming velocities of two populations of *Paramecium caudatum* specified by curvature of the observed swimming course (filled circles: fast and *straight* swimmers, curvature < 0.10/mm; open circles: slow and *curved* swimmers, curvature > 0.85/mm). The lines give the calculated velocities[5] at 6 different angles between vertical downward and vertical upward on the assumption that only the invariant velocity and sedimentation determine the observed velocity (continuous line: of straight swimmers; broken line: of curved swimmers. It is seen that (1) the offset from the "no-gravikinesis" line is larger in curved swimmers approximating full cancelling of the sedimentation rate, (2) gravikinesis acts to reduce downward swimming and to raise upward swimming rates, (3) the size of the gravikinetic component of velocity decreases from vertical toward horizontal orientation. Modified after Ooya et al. (1992), with permission

density to 1.033 g/cm^3 (viscosity relative to water, 1.12; 24.5°C), which reduces the density decrement between *Paramecium* cytoplasm and freshwater from 0.04 to 0.007 g/cm^3, that is, to less than 20% of its original value (see 2.1). According to the statocyst hypothesis, reduction in gravity-induced pressure difference attenuates the gravikinetic response. Figure 16 shows that the slope of gravity-induced change in invariant propulsion per angular reorientation was reduced in the Percoll-cells, straight as well as curved swimmers. From this, Ooya et al. (1992) have concluded that in *Paramecium*, the cell membrane is the primary candidate for gravireceptor, and pressure difference across the membrane rather than an absolute hydrostatic pressure increment is the crucial signal for gravireception (see 4.2). Further neutral-buoyancy experiments are now awaited in ciliates which may even more substantiate this important conclusion.

8.4.1.5 Nonswimming Locomotion

The "gliding" of *Loxodes* along substrate surfaces is a preferred type of locomotion under steady-state conditions, and its orientation and velocity can be adequately measured (Bräucker et al. 1992). For investigations of a possible gravikinesis of gliding cells the sedimentation rate is difficult to determine because wall effects (Wu 1977) may reduce the rate of a sedimenting specimen. Bräucker et al. (1992) observed that the gliding rate in *Loxodes* is independent of the cell's orientation in space suggesting complete compensation of the sedimentation rate whatever its size (see, in addition, 8.4.3). The sedimentation rate during gliding locomotion of *Loxodes* remains to be established during step transition of horizontally moving cells from normal
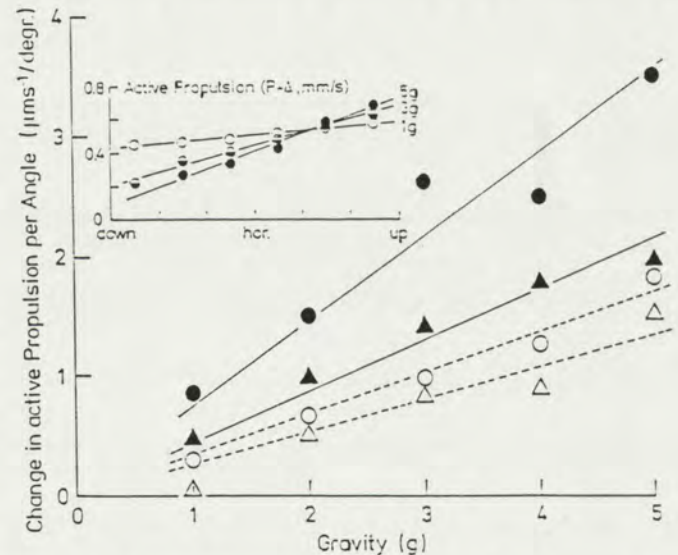
Fig. 16. Evidence for external-membrane based gravireceptor in *Paramecium*. Isolation of active propulsion from swimming velocity and sedimentation rate for 6 different orientations of curved swimmers (between vertical down and vertical up) gives a linear slope, which rises with gravity increasing from 1 g to 5 g (**inset**). This slope was plotted as a function of gravity (g) for *curved swimmers* (filled symbols) and *straight swimmers* (open symbols) in Dryl's solution (circles) and Percoll solved in Dryl's solution (1.033 g/cm^3; triangles). It is seen that the increase in gravikinetic response was proportional to gravity, and was depressed in the Percoll solution, in particular in the curved swimmers. The residual gravikinetic sensitivity in the Percoll solution is due to the remaining 18% of normal gravitational pressure difference across the lower membrane, or results from an additional unknown mechanism of gravireception. Modified after Ooya et al. (1992), with permission

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[5] If, and only if, the swimming rate of a ciliate is assumed to include no gravity-induced kinetic component (Δ), the propulsion as unaffected by gravity (= invariant velocity, P) results from the arithmetic mean of the vertical downward and upward swimming velocities (V_D, V_U). An observed velocity in arbitrary direction is then determined by

$$V = \sqrt{P^2 - S^2 \sin^2\theta} - S\cos\theta \,, \quad \text{(Ooya et al. 1992)}$$

where θ is the inclination angle of V (see Fig. 9). Assuming that a gravikinetic component of velocity (Δ) adds to P, this equation is equivalent to equation 4.

Fig. 17. Circular distributions of velocity and orientation of *Paramecium caudatum* before and during free fall in a 4.6 s-drop tower (4×10^4 g). Reference cells were kept in the horizontal chamber, showing non-sided distributions of velocities and orientation (right column). Slow turning of chamber to the vertical at 1 g induced a gravitational bias in velocity and orientation within 1 min. During two periods of free fall (1.5 to 3 s; 3 to 4.5 s after start), the distributions of velocity and orientation were increasingly randomized

Table 2

Gravikinesis (Δ) as calculated using the vertical velocity down (V_D) and up (V_U), and the sedimentation rate (S) of ciliates (from different experiments and authors). Values of kinesis during downward and upward motion (Δ_D, Δ_U) were determined using the velocity as unaffected by gravity (P; Machemer et al. 1991). All velocities in μm/s

Species	± Degree from vertical	V_D	V_U	S	Δ	P	Δ_D	Δ_U	n
Paramecium caudatum [1]	15	639	500	89	**-20**	568		·	63, 203
	45	641	502	89	**-20**	568	-14	-25	221, 487
P.c. [2]	90	843	808	84	**-67**	834	-75	-58	364, 674
P.c. [3]									
straight path	30	807	639	119	**-35**				2000
curved path	30	615	523	119	**-73**				2000
P.c. [4]									
norm. swimmers	45			89	**0 to -70**	850			18310
fast swimmers	45			89	**0 to -90**	1850			27279
Didinium nasutum [1]	45	1504	1401	90	**-39**				86, 286
Loxodes striatus [5]	0	169	169	49[7]	**-49**[7]	169	-49[7]	-49[7]	835, 816
L.s. [6]	15	159	153	48[7]	**-47**[7]	156	-45[7]	-45[7]	2402, 2152

[1] Machemer et al. (1992)
[2] Machemer et al. (1991)
[3] Ooya et al. (1992, extracted from their Figs. 3 and 4)
[4] Machemer et al. (1993)
[5] Bräucker et al. (1992)
[6] Machemer-Röhnisch et al. (1993)
[7] on the so far unproven assumption that sedimentation rate of gliding cell corresponds to sedimentation rate of free suspended cell
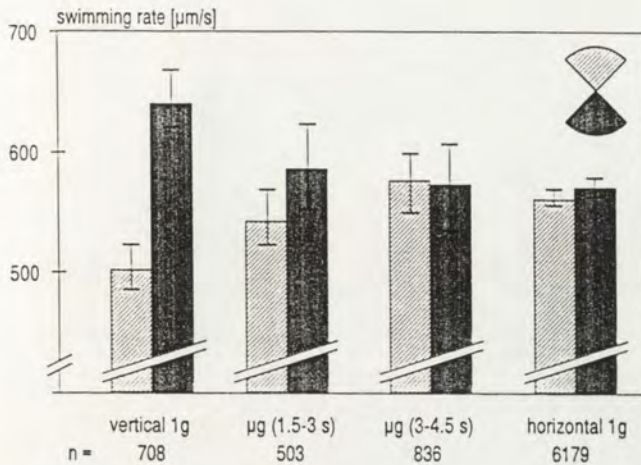
Fig. 18. Quantitative changes in gravity-induced swimming velocity of *Paramecium* (data shown in Fig. 17). At 1 g, the rates of upward swimming were significantly smaller than the rates of downward swimming (medians, 95% confidence range). Following step-transition to weightlessness, these differences gradually subsided in the course of 4.5 s, and the velocities were inseparable from those observed during horizontal orientation at 1 g of the same cell population. The same 90°-sectors (inset) of the observed field were used for evaluations throughout. Machemer at al. (1992) with permission

gravity to weightlessness. Populations of *Loxodes* were found to occur in equal proportions moving vertically upward or downward. This orientational response, together with neutralization of sedimentation, was termed a "neutral gravitaxis" (Machemer-Röhnisch et al. 1993).

8.4.2 Microgravity Experiments

A preliminary exploration of the behaviour of *Paramecium* during a 35 m free fall (10^{-2} g; Mogami et al. 1988) showed reduction in velocity of upward swimming single specimens after transition to 2 s of weightlessness. Using a drop tower of 110 m free fall at 10 Pa (10^{-4} g) and populations of about 100 cells, the gravity bias of velocity and orientation relaxed in the course of 4.5 s (Fig. 17). The direction coefficient (r) dropped from 0.200 ($r_0 = r$) at 1 g to 0.136 at the end of weightlessness (Machemer et al. 1992) indicating that full random orientation of *Paramecium* was not achieved within a few seconds of weightlessness. With weightlessness continuing over several minutes in a sounding rocket experiment ($x10^{-6}$ g; Fig. 19B), r-values centered about 0.08 (extremes: 0.2 and 0.02; Hemmersbach et al. 1993).

Quantitative evaluation of velocities in *Paramecium* before and during transition to weightlessness confirmed the difference between upward and

downward swimming rates at 1 g (Fig. 18, Machemer et al. 1992). The difference did not immediately disappear with the step-type removal of the gravity bias, but attenuated in the course of 4.5 s. This suggests a slow relaxation time constant of the gravikinetic response. Because electromotor coupling events involve fractions of a second for minor shifts in membrane potential (Machemer 1975), the observed slow relaxation of gravikinesis may be a cue of the nature of the receptor mechanism. The final free-fall values of previously upward and downward swimming rates coincide suggesting that *Paramecium* showed the value of velocity (P) as unaffected by gravity. This P-value was the same as the median rate of swimming in horizontally oriented cells at normal gravity (Fig. 18). The data suggest that the velocity in well equilibrated, horizontally swimming cells is a good measure of the important value of invariant propulsion (P; see 8.1.1).

8.4.3 Hypergravity Experiments

Raising gravity by linear acceleration or centrifugation is quite useful for investigating gravireception. Behavioural consequences of gravity, which are subtle and hard to document under natural gravity, may be potentiated during hypergravity. The sedimentation rate of free floating immobilized cells rises in proportion to gravity according to Stoke's law, but the unstirred layer of .water near the walls of the experimental chamber (8.3.3) may interfere with the sedimentation-gravity relationship. Ooya et al. (1992) confirmed exact proportionality up to 5 g for *Paramecium*. These authors documented an increase in gravikinesis with gravity, which was most pronounced in slow cells swimming in arcs (Fig. 16). At 1 g sedimentation was 119 μm/s and mean gravikinesis was -73 μm/s (Table 2); at 5 g and 5-fold increase in sedimentation rate, their plots (Ooya et al. 1992, Fig. 4) indicate a gravikinetic rate of -456 μm/s corresponding to a rise in gravikinesis by a factor of 6.2.

Application of hypergravity in *Loxodes* revealed a difference in gravikinetic responses of vertically upward and downward gliding cells. Upward velocities were maintained up to 5.4 g, whereas downward velocities steadily rose with the gravitational force (Machemer-Röhnisch et. al. 1993). These data indicate that the value of gravikinesis and, implicitly, the signal generated by
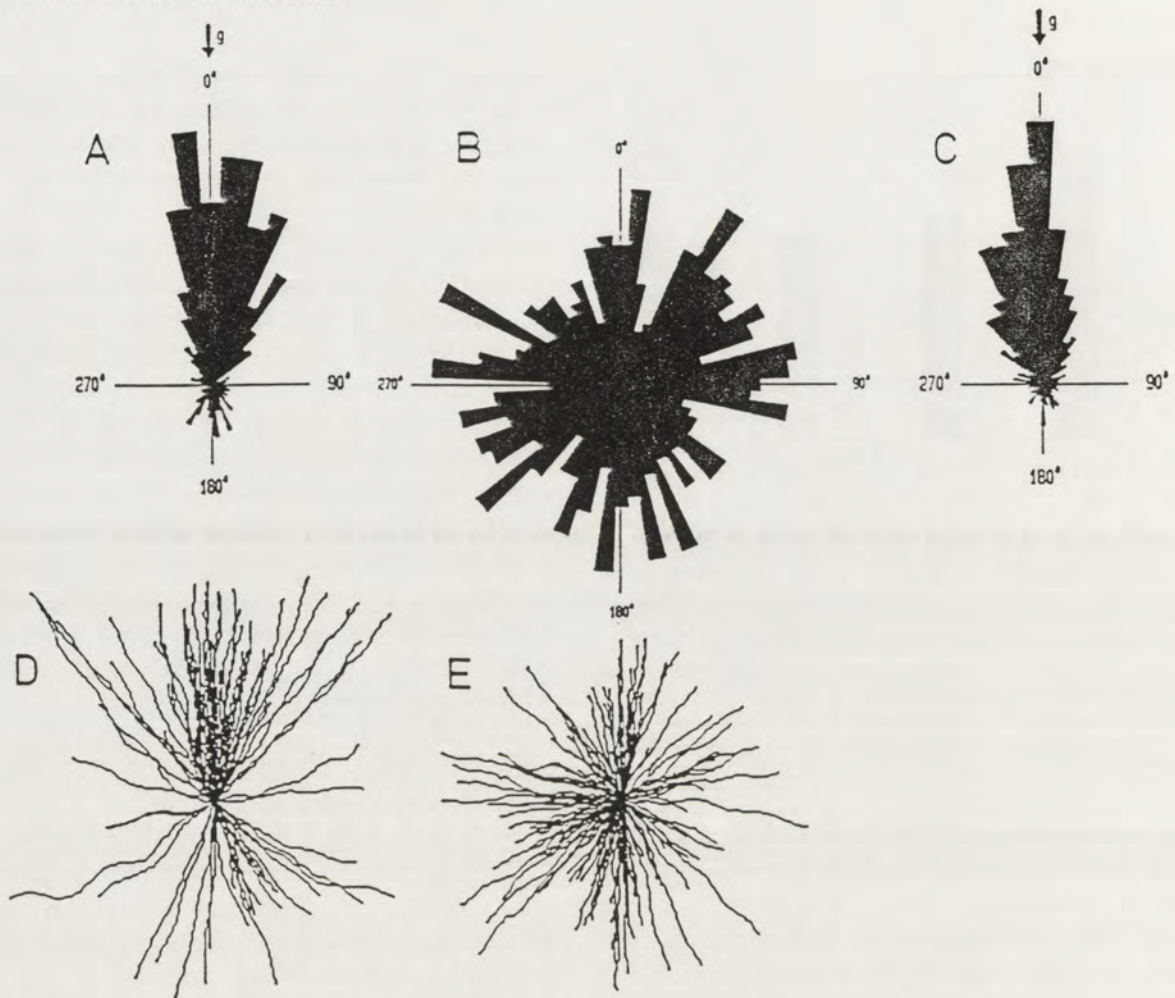
Fig. 19. Orientation and movement of *Paramecium (aurelia* group) at normal gravity and during free fall inside a sounding rocket rising to 250 km altitude. **A.** On ground 5 min before launch; n = 883. **B.** 5 min of weightlessness (4x10⁻⁶ g); n = 863. **C.** 45 min after landing; n = 2509. **D.** Swimming traces on ground. **E.** Swimming traces during weightlessness. Hemmersbach et al. (1993), with permission

the gravireceptor, differs during upward and downward locomotion under artificially raised gravity, even though it may be the same in all directions at normal gravity (Bräucker et al. 1992, Table 2). Hypergravity induced the neutral gravitactic response of *Loxodes* (8.4.1.5) to shift from the line of resultant acceleration vector by a counterclockwise angle (5.4 g: 60°). This error in graviorientation was reproducible with rising and decreasing hypergravity (Machemer-Röhnisch et. al. 1993). Observations of hypergravity-induced angular offset have also been reported from *Paramecium* (Hemmersbach-Krause unpublished). Running hypergravity experiments in forward and backward sequence (1 g→hyper-g; hyper-g→1 g) did not affect the observed velocities and orientations of *Loxodes* (Machemer-Röhnisch et. al. 1993) suggesting that the graviresponse does not adapt on short term in agreement with findings in *Paramecium* (Ooya et al. 1992).

9. CONCLUSION AND PERSPECTIVE

An overview of the current literature on gravireception and graviresponses in ciliates leads to a clear result: the statocyst hypothesis, in electrophysiological reinterpretation (Fig. 20), gives the theoretical framework, into which experimental data can be fitted with ease, and using this paradigm, new experiments may be designed which ask precise questions. So far, no alternative model of gravireception in the free swimming cell is available with similar predictive value. At the same time, physiological and exclusively physical mechanisms of graviresponses are likely to coexist in ciliates. Evidence is available suggesting that various strategies are being used by ciliates to guide their ways in a world, where "Up" and "Down" are crucial
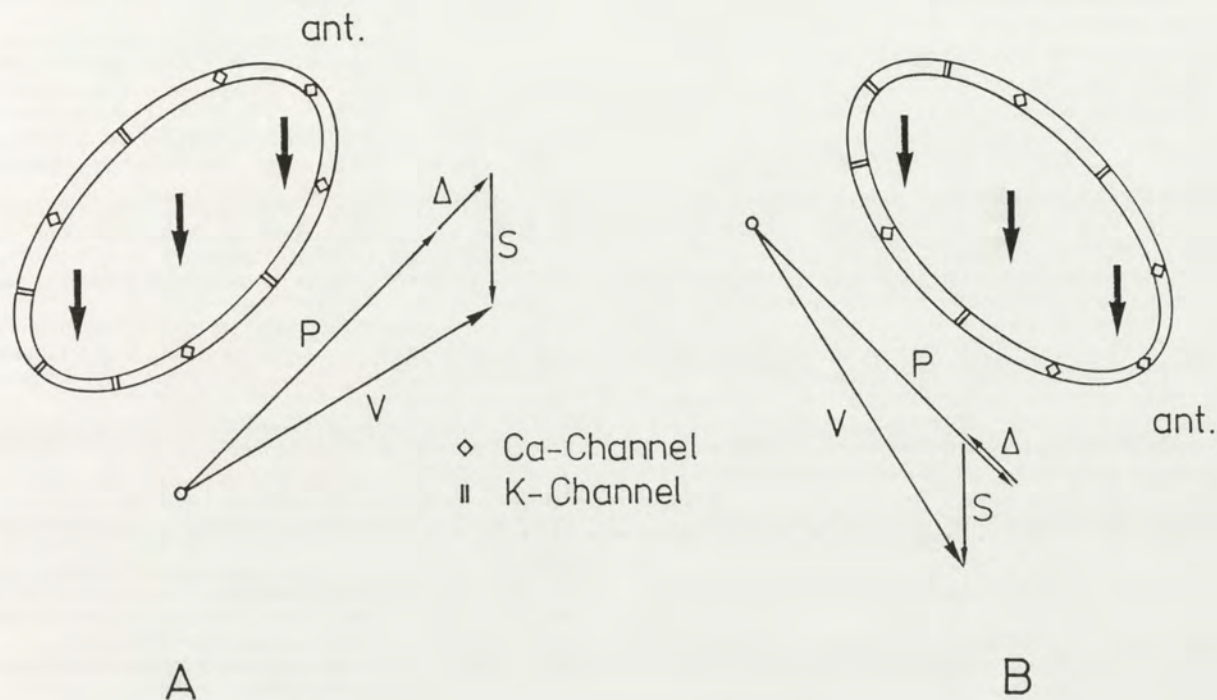
Fig. 20. Simplified model of gravitransduction and kinetic response in the ciliate organism. A cell is thought to swim upward (A) or downward (B) in arbitrary direction (ant., anterior cell end). Cytoplasmic density generates differential pressure across the actual lower membrane activating, by deformation of a sensitive intramembranous structure, mechanically sensitive membrane channels. **A.** During upward locomotion the posterior membrane is deformed; here gravireceptor K-channels prevail leading to hyperpolarization and augmentation of the ciliary beating rate. The invariant rate of propulsion (P) grows by a gravity-induced component (Δ) and combines with the rate of sedimentation (S) to produce the observed direction and velocity of the cell (V). **B.** In the downward swimming cell gravity deforms the anterior membrane activating anteriorly prevailing gravireceptor Ca-channels. The resulting depolarization slows down steady ciliary propulsion, and the swimming rate (V) results after subtraction of Δ from P with S maintained. The sign and size of the gravikinetic response (Δ) determine, together with sedimentation and possible gravity-induced mechanisms of reorientation, the net locomotion of cells with respect to the gravity vector

cues for survival. The complexity of interactions between gravity and the cell, and the subtleness of the stimulus have been a challenge for generations of researchers, and there is reason to expect that this will continue in the foreseeable future. Modern methods and advanced tools of data evaluation support the analysis of graviresponses but, at the same time, highly sensitive instruments have also raised the experimenter's awareness of the conditions, which affect the behaviour of the organism.

A major goal of this review is practical: fostering the understanding of the ciliate organism as a model for investigations of cellular gravireception and graviresponses, and calling attention to possible physical environments of gravistimulation. Knowing and gaining command on such conditions, or neglecting them, may decide on the size of experimental "noise" or, eventually, isolation or masking of a graviresponse. Much methodical work is still to be

done. While an exhaustive description of the signalling pathway of gravity is the ultimate goal, direct measurements of receptor currents and potential responses to gravity are demanding tasks, and success may be still far ahead. We have pointed out that gravity is a weak stimulus and difficult to handle. Only few types of ciliates have so far been accessible to intracellular electrophysiology, and those few may not altogether qualify for investigations of gravireception. Individual variability in responsiveness to the gravity stimulus is a restriction to conclusions drawn from experimental work on single cells. Experiments on large numbers of cells with the potential to produce relevant biophysical information must be designed and developed to maturity. In the near future, a host of increasingly sophisticated behavioural analyses of graviresponses may have a pacemaking function for the understanding of gravitransduction in ciliates.

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Correction

1. The citation "Baba (1991) is incomplete; the correct writing is "Baba et al. (1991). Correspondingly, in the References should be: Baba S.A., Tatematsu R., Mogami Y. (1991) A new hypothesis concerning graviperception of single cells, and supporting simulated experiments. Biol.Sci.Space 5: 290-291 (in Japanese).

2. The citation od Pfeffer (1904) is to be deleted from the text and in the References.

ACTA
PROTOZOOLOGICA

Evidence for Growth Factors which Control Cell Multiplication in *Tetrahymena thermophila*

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Summary. The ciliate *Tetrahymena thermophila* was grown in a standard synthetic nutrient medium that supports doubling times of about 2 h. Cell multiplication was investigated as a function of initial cell density and addition of either certain chemical compounds, or cell-free extracellular fluid. In cultures inoculated at 750 or more initial cells per ml the cells initially thrived and multiplied but below this density the cells soon died. Addition of extracellular fluid or either hemin, phospholipids or bovine serum albumin, egg albumin, trypsin, or soy bean trypsin inhibitor, however, saved these cells from dying and supported cell proliferation. We have also investigated the cloning efficiency of the cells as a function of culture volume of the synthetic medium. In 1 ml portions of standard synthetic medium the cloning efficiency was 0%, but in volumes of 1 μl it was 90%. We suggest that *Tetrahymena thermophila* produces and releases growth factors that stimulate the cells to leave the lag phase and start cell multiplication. Certain compounds like hemin, phospholipids and certain proteins can substitute for these growth factors.

Key words. Synthetic nutrient medium, cell multiplication, cell proliferation, initial cell density, growth factors, hemin, phospholipids, proteins, *Tetrahymena*.

INTRODUCTION

The first recipe for synthetic nutrient medium for *Tetrahymena thermophila* is more than 40 years old (Kidder and Dewey 1951). This type of medium has been used for gaining insight into nutrient requirements (Holz 1973), metabolic pathways (Kidder 1967), uptake mechanisms (Dunham and Kropp 1973) and reactions between inorganic and organic molecules in the medium (Hutner 1972). In spite of the fact that it was possible to maintain doubling times and final cell densities comparable to those obtained in the best complex medium, the synthetic medium are deficient in at least one respect: single cells in 1 ml cultures die shortly after being transferred to such a medium (Christensen et al. 1992).

We have recently shown that *T. thermophila* grown under nutritional stress situations need various compounds not required at initial densities of more than 1,000 cells per ml. The stress conditions consist of growth at low population density (Schousboe et al. 1992) or growth at low ambient nutrient concentrations (Christensen et al. 1992). These results agree with the notion that the cells produce and release compounds with effects like those of growth factors (Ghiladi et al. 1992). Here we present further detailed evidence for these views.

MATERIAL AND METHODS

Cells: The following cell lines of *Tetrahymena thermophila* were used: wildtype inbred strain B 1868-III (Orias and Bruns 1976); the mutant strains MS-1, secreting lysosomal enzymes at low rates (Hünseler et al. 1987); II8G, defective in food vacuole formation (Tiedtke et al. 1988); and SB 281, releasing no mucocysts (Orias et al. 1983).

Nutrient medium: Cells were grown either in a complex medium, *PPYS*, or in a standard synthetic nutrient medium, *SSM*. *PPYS* is a solution of 0.75% proteose peptone (Difco Laboratories, Detroit, Michigan, USA) enriched with 0.75% yeast extract (Difco) and salts. *SSM* consists of 19 amino acids, 4 nucleosides, glucose, 7 vitamins, salts, and citrate (Szablewski et al. 1991). In both cloning and mass culture experiments *SSM* was diluted in the ratio of 1:1 with TRIS/HCl buffer (pH 7.5). When testing the presence of stimulatory compounds in extracellular fluid, we replaced the buffer with extracellular fluid, keeping the concentrations of the nutrients in the medium constant. Cells requiring special conditions for growth and multiplication were grown in media modified to fulfill these conditions: mutant strain II8G was grown in medium enriched with iron and copper salts and folic acid as prescribed for this mutant (Tiedtke et al. 1988). Chemicals were from Sigma Chemical Co., St Louis, Missouri.

Cultures: Stock cultures were grown in 2 ml portions of standard synthetic medium, *SSM*. Experimental cells were grown in 10 ml portions of *SSM* in conical flasks for 20 h, transferred to 10 mM TRIS/HCl buffer (pH 7.5), centrifuged for 3 min at 800x*g* and resuspended in the buffer. This procedure was repeated three times and resulted in more than a 10^4-fold dilution of the extracellular fluid. The cells were then divided into three batches and used as follows: i) for preparation of extracellular fluid, ii) for multiplication analysis in 10 ml portions of *SSM* where effects of initial cell density and presence of hemin, phospholipids, proteins or extracellular fluid were studied, and iii) for cloning analysis in various volumes of either *PPYS* or *SSM* or *SSM* enriched with either hemin, phospholipids or proteins. The cells were grown at 37°C.

Preparation of cell-free extracellular fluid: Cells were starved in 10 ml TRIS/HCl buffer (pH 7.5) in conical flasks for 0-5 min and 5 h at a density of 50,000 cells per ml. After that, a sample of a culture was transferred to a centrifuge tube and placed on top of 2 ml of a solution of 10% Ficoll and precipitated for 10 min at 3000x*g*. The Ficoll solution was sterilized by filtration and used at 4°C. The *cell-free* supernatant, containing *no cells*, was removed and used in the experiments. The Ficoll has a high specific weight and high viscosity and prevents the cells from swimming back up. To certify that no cells were left in the extracellular fluid a sample was placed in a small Petri dish and checked under a stereo microscope.

Cloning procedures: After centrifugation and resuspension in the buffer single cells were transferred to two different sizes of cultures, 1 ml or 10^{-3} ml (1μl). *One ml cultures*: single cells were transferred to either *PPYS*, *SSM* or *SSM* enriched with either hemin, phospholipids or proteins with a fine pipette (see Schousboe et al. 1992). *One μl cultures*: single cells were suspended in either *SSM* or *PPYS* in test tubes at a density of 1,000 cells per ml. By means of a 10 μl Hamilton pipette droplets of 1μl from the test tube were placed on the top of paraffin oil placed in a glass Petri dish. The droplets sank to the bottom of the dish and the paraffin oil prevented evaporation of the medium. The dish had a diameter of 7 cm, the paraffin oil was autoclaved at 121°C for 45 min and reached a height of 2 mm in it. Only droplets containing a single initial cell were kept under observation.

Cell densities in test tubes and droplets were recorded every day. In most cases many cells (1,000) were present already after the first 24 h. In all cases droplets and tubes were kept under observation until day 4.

Compounds added to *SSM*: Hemin and asolectin, a crude preparation of phospholipids, were used at final concentrations of 7.5 μM and 50 μg per ml, respectively (see Schousboe et al. 1992 and Christensen et al. 1992). Bovine serum albumin, egg albumin, trypsin and soy bean trypsin inhibitor were used as sources of proteins. They were dissolved in redistilled water, sterilized by filtration and used at final concentrations of 50 μg per ml.

Cell counting: Culture samples were enumerated in an electronic particle counter (see Christensen et al. 1992).

All experiments were repeated more than 10 times with similar results.

RESULTS

Mass cultures

Initial cell density is an important parameter in cell multiplication of *Tetrahymena* in synthetic medium. Figure 1 shows the number of cell doubling of *T. thermophila*, wildtype, as a function of time and number of cells transferred into *SSM*. In cultures having 750 or
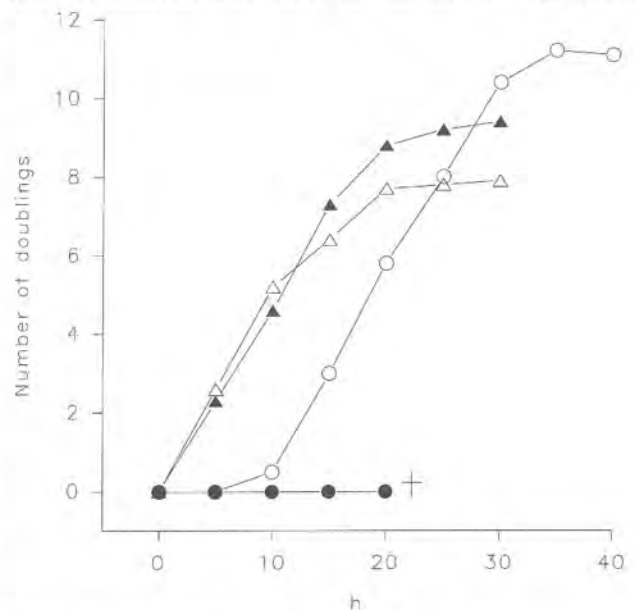
Fig. 1. The number of population doubling of *Tetrahymena thermophila*, wildtype, as a function of time and initial population density in standard synthetic medium, *SSM*; ● : 250 and 500 cells per ml, ○ : 750 cells per ml, ▲ : 1000 cells per ml, △ : 2500 cells per ml. All cultures were observed for the whole duration of the experiment. Final cell densities were in all cases about 500,000 cells per ml. Differences in the number of cell doubling are due to differences in initial cell densities. The cells died, if they had not initiated multiplication after about 20 h
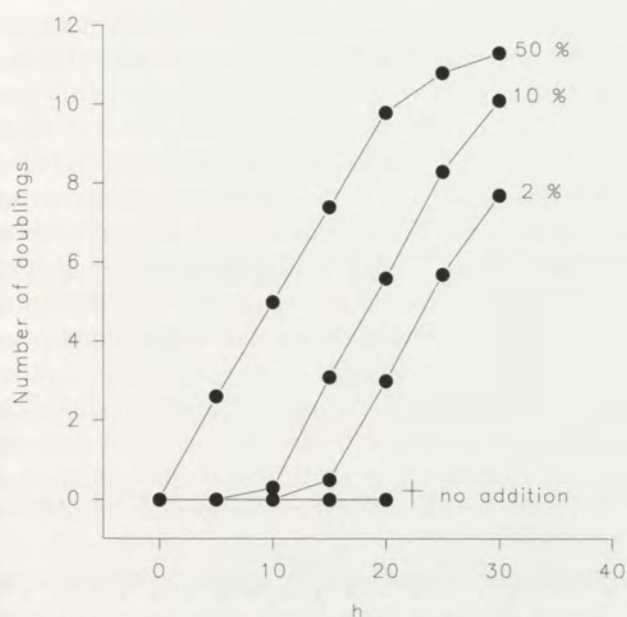
Fig. 2. The number of population doubling of *Tetrahymena thermophila*, wildtype, as a function of time and percentage of extracellular fluid in standard synthetic medium, *SSM*. All cultures were inoculated at 250 cells per ml. The cells died, if they had not initiated multiplication after about 20 h

Fig. 3. The number of population doubling of *Tetrahymena thermophila*, wildtype, as a function of time and added chemical compound in standard synthetic medium, *SSM*. All cultures were inoculated at 250 cells per ml; Δ : *SSM* + phospholipids (50 µg per ml); $\bigcirc$: *SSM* + hemin (7.5 µM); and $\square$: *SSM* + bovine serum albumin, BSA, (50 µg per ml)

more initial cells per ml the cells thrive, whereas 250 and 500 cells per ml die within 20 h. One thousand or more cells per ml have a short lag phase (about 1 h); 750 cells per ml have a lag phase lasting about 10 h.

Extracellular fluid is also an important parameter for cell proliferation in synthetic medium. Figure 2 shows the number of cell doubling of *T. thermophila*, wildtype, as a function of time and concentration of added extracellular medium where cells were starved for 5 h. In all cases the initial cell densities were 250 cells per ml. Without any addition of extracellular fluid the cells do not multiply. If extracellular fluid is added in the ratio of 1 portion of *SSM* to 0.02 portion of extracellular fluid

(to achieve 2% of extracellular fluid in the medium) the cells multiply with a lag phase of about 15 h. If the extracellular fluid amounts to 10% of the volume, the cells have a lag phase lasting about 10 h before multiplying, and where extracellular fluid is 50% of the media's volume the cells have a short lag phase, repeating the growth curve representing 2,500 cells per ml.

Hemin or phospholipids or proteins also initiate cell proliferation. Figure 3 shows the effects of these compounds on cell multiplication in the assay system used for testing effects of extracellular fluid. Cells in *SSM* enriched with these compounds multiply after a short lag phase (0-1 h), repeating the growth curve representing 2,500 cells per ml.

It should be noted, that if the cells survive and start multiplication then they multiply at the same doubling times (about 2 h) and reach the same final cell densities irrespective of the duration of the lag phase.

Cloning

Results of cloning experiments in various volumes of *PPYS*, *SSM* and *SSM* enriched with either hemin, phospholipids or proteins, are listed in Table 1. *T. thermophila*, wildtype, in 1 ml portions do not form clones in *SSM* unless it is supplemented with hemin, phos-

Table 1

Cloning efficiency in 1 µl or 1 ml of complex medium, *PPYS*, standard synthetic medium, *SSM* and *SSM* supplemented with either hemin (H), Phospholipids (PL), bovine serum albumin (BSA), egg albumin (EA), soy bean trypsin inhibitor (SBTI), or trypsin. As an example, the expression "29/32" indicates that 29 out of 32 single cells formed clones. Hemin was used at 7.5 µM and phospholipids and proteins at 50 µg per ml. The dashes indicate conditions which were not tested since the cloning efficiency was high in 1 ml portions

Culture			SSM supplemented with					
volume	SSM	PPYS	H	PL	BSA	EA	Trypsin	SBTI
1 ml	0/30	29/30	14/15	15/15	12/15	11/15	19/20	16/20
1 µl	29/32	15/15	-	-	-	-	-	-

pholipids or the proteins bovine serum albumin, egg albumin, soy bean trypsin inhibitor or trypsin or grown in *PPYS*. In 1 µl portions of *SSM*, however, the cells have a high probability to form clones. Thus the cloning efficiencies of *T. thermophila* in *SSM* are culture volume dependent. The presence of paraffin oil in test tubes with one ml of either *SSM* or *PPYS* does not affect the cloning efficiencies (data not shown).

DISCUSSION

Many workers have shown that a wide variety of compounds are released from *Tetrahymena*. These include hydrolytic enzymes (Tiedtke et al. 1992), mating pheromones (Adair et al. 1978), transcription inhibiting factors (Andersen et al. 1980), mucocysts and accompanying compounds (Maihle and Satir 1986) and proteins without ascribed functions (Suhr-Jessen 1987). Our results suggest that *Tetrahymena* also release unknown compounds with effects on cell proliferation. Also known groups of compounds (tetrapyrroles, phospholipids and proteins) have similar effects on cell proliferation (Schousboe et al. 1992 and Christensen et al. 1992). The cell-produced factors appear to be different from those added by us in that they are active at lower concentrations.

Our experiments show that cell-free media samples of a TRIS/HCl buffer, in which cells have been starved, improve cell proliferation and reduce lag phases in a concentration dependent fashion, Figure 2. As controls, we have also made experiments with cell-free media samples, where cells have been exposed to starvation conditions for a period of 0 to 5 min. In these cases we did not see any stimulatory effect on cell multiplication. Furthermore, we have shown that single cells in 1 µl of standard synthetic medium (corresponding to an initial cell density of 1,000 cells per ml) form clones with high probability. These observations indicate that components from dead cells - or physical contacts between the cells - are not responsible for the stimulation. Therefore, it seems that a small volume of growth medium allows the single cell to build up sufficient concentrations of one or more molecules stimulating its own growth and multiplication. In larger volumes these molecules may become too diluted to be effective.

The ideas presented here may explain why initial cell densities are decisive for the fate of *T. thermophila* in standard synthetic medium. The amounts of growth stimulating factors released at low initial cell densities may be insufficient for stimulation of the cells. However,

at higher cell densities the amounts of these molecules may be sufficient to support cell proliferation. This idea may also explain why cells exposed to starvation conditions for 4 h can multiply when inoculated at 250 cells per ml in synthetic medium as previously reported (Christensen et al. 1992): the inoculation volume needed to transfer cells to the growth medium may contain sufficient amounts of growth stimulating compounds which had been released during the period of starvation.

We have previously shown that certain porphyrins, including hemin, as well as phospholipids, improve proliferation under nutritional stress and conditions of expected low cloning efficiencies in standard synthetic medium (Schousboe et al. 1992 and Christensen et al 1992). Here we show that these compounds, in addition to certain other proteins, stimulate cell growth and multiplication in mass culture experiments where the cells are inoculated immediately after the extracellular medium had been diluted more than 10^4-fold by centrifugation and resuspension in a TRIS/HCl buffer. These data, combined with results obtained with extracellular fluid and "micro-cloning", seem to indicate: i) that *Tetrahymena thermophila* produce and release growth stimulating compounds into the growth medium; ii) that the compounds are responsible for cell proliferation in standard synthetic nutrient medium; iii) at low initial cell densities we can see that exogenous compounds as hemin, phospholipids and certain proteins can substitute for cell-produced stimulatory compounds which may be present, but at ineffective concentrations; iv) stimulation is expressed neither in changes of doubling times, nor in attainment of higher final cell densities, but in the rate with which the cells leave the lag phase and start growth and multiplication. We choose to name these stimulatory compounds *growth factors*, and point out that they appear to represent a group of compounds different from the well-known nutritionally required components.

PPYS supports cell proliferation at low initial cell densities. This may mean that the complex medium contains growth factors or growth factor-substituting molecules, e.g. proteins. In *SSM* these compounds are missing and the cells are forced to adapt or "condition" the medium by releasing their own growth factors. Thus, survival, growth and multiplication are dependent on cell-produced growth factors in synthetic medium.

There are several possible mechanisms behind release of compounds from *Tetrahymena*. Molecules leave the cells either via lysosomes (Tiedtke et al. 1992), mucocysts (Maihle and Satir 1986), or egestion of food

vacuoles to mention a few. To investigate possible connections between some of these mechanisms and the release of growth factors, we have made experiments with the mutant cell strains II8G (lacking food vacuoles), MS-1 (releasing lysosomal enzymes at low rates) and SB 281 (releasing no mucocysts). The observed patterns of growth and multiplication in both cloning and mass culture experiments were similar to those presented in Figures 1, 2 and 3 and Table 1. This seems to exclude the possibility that growth factors are released together with lysosomal enzymes and compounds from mucocysts. Furthermore, food vacuoles appear to play no part in either utilization or release of the growth factors.

Tanabe et al. (1990) have reported on a growth factor from a mutant *Paramecium tetraurelia*. When this factor is added to the mutant, it will recover the multiplication rates characteristic of the wildtype. In our case the growth factor is so far unknown, but it is apparently secreted by wildtype cells. Cells in synthetic medium seem to require the presence of a growth factor.

Previously, Kidder and Dewey (1951) reported that it was necessary to inoculate *Tetrahymena* cultures in synthetic medium with not less than 1% of the new culture volume. This concurs with the idea that the cells somehow change their medium making it fit for cell survival and multiplication. Lilly (1967) and Lilly and Stillwell (1965) have reported on the effects of growth factors on multiplication of *T. pyriformis* in conditioned medium. Their results indicate that such compounds may be present, but the reported difference between control and experimental cultures was small. In view of our results it is obvious, that their initial cell densities (20,000 cells per ml) were too high to reveal any significant effect.

The experiments presented here indicate that a variety of compounds, including hemin, phospholipids etc., can initiate cell multiplication in synthetic medium. These compounds may act as signals for cell multiplication. They may do so by acting on the cell surface, in the cytoplasm or inside the cell nucleus. Furthermore, the cells themselves appear to release these or other factors, by what looks like an autocrin process, to stimulate their own cell division.

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ACTA
PROTOZOOLOGICA

L'Ultrastructure du Cilié *Myxophyllum steenstrupi* (Stein, 1861) Parasite de Mollusques Pulmonés et la Famille des *Thigmophryidae* Chatton et Lwoff, 1923

Ultrastructure of Ciliates *Myxophyllum steenstrupi* (Stein, 1861) Parasite of Molluscs and Family of *Thigmophryidae* Chatton et Lwoff, 1923

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Résumé. Une comparaison est faite entre les ultrastructures corticale et buccale des ciliés *Myxophyllum* (endocommensal de Pulmonés), *Myxophthirus* (endocommensal de Bivalves marins) d'une part, *Conchophthirus* (endocommensal de Bivalves d'eau douce), Thigmotriches (endocommensaux surtout de Mollusques marins), d'autre part.

Chez tous est différencié un cortex locomoteur et un cortex thigmotactique. Le cortex locomoteur est formé de cinéties à monocinétides, sauf chez *Myxophthirus* où les cinétosomes y sont, le plus souvent, appariés et cilifères.

Chez *Myxophyllum* un fin cordon microfibrillaire à noeuds longe la droite des cinétosomes, alors que chez *Myxophthirus* y sont présents 2-3 microtubules longitudinaux parallèles à chaque cinétie, comme chez les Thigmotriches (*Proboveria*) et que chez les *Conchophthirus* il y a des rideaux de microtubules sous-cinétiens.

Les monocinétides des cinéties corticales locomotrices ont les dérivés fibrillaires tangentiels des Scuticociliés, dont un tractus transverse avec le rideau des microtubules transverses.

Le cortex thigmotactique est toujours constitué de dicinétides dont le cinétosome postérieur porte les mêmes systèmes fibrillaires tangentiels que ceux associés aux cinétosomes isolés, le cinétosome antérieur étant pourvu de quelques microtubules transverses et d'un tractus transverse. Alors que chez *Myxophyllum*, *Myxophthirus*, *Conchopthirus* les 2 cinétosomes des dicinétides sont décalés l'un par rapport à l'autre et accompagnés chacun d'un sac parasomal, chez les *Proboveria* les cinétosomes sont groupés en file, avec un seul sac parasomal médian à droite par groupe et le cinétosome moyen porte une fibre cinétodesmale comme le cinétosome postérieur. Un thigmoplasme à base de saccules et de vésicules est toujours différencié au niveau de la zone thigmotactique. L'emplacement de la ligne antérieure de suture des cinéties délimitant les 2 faces principales est toujours renforcée d'un cytosquelette de microtubules inclus dans une matrice.

Chez *Myxophyllum* et *Myxophthirus* il y a de nombreuses mitochondries éparses dans l'endoplasme alors que le chondriome de *Conchophthirus* et des Thigmotriches (*Ancistrum*, *Proboveria*, *Boveria*) est essentiellement formé de gros et longs chondriocontes inclus dans les crêtes ectoplasmiques.

Dans tous les cas l'organelle paroral est une stichodyade associée à un réseau microfibrillaire à noeuds.

Chez *Myxophyllum* et *Myxophthirus* l'organelle adoral est unique et formé de rangées de cinétosomes dont certains portent des fibres microtubulaires postciliaires. Les 3 organelles adoraux du plafond de la cavité buccale de *Conchophthirus* sont différents de ceux des Thigmotriches formés de la juxtaposition de 2-3 rangées de cinétosomes dont tous portent des microtubules postciliaires.

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Il parait convenable de réunir les *Myxophyllum* et *Myxophthirus* avec *Thigmophrya* dans la famille des *Thigmophryidae* incluse dans les Scuticociliés *Philasterina*, de considérer les *Conchopthiridae* comme une famille distincte des Scuticociliés *Pleuronematina* et, en l'absence de données ultrastructurales sur l'ensemble *Pleuronema-Histiobalantium* d'accepter un sous-ordre *Thigmotrichina*.

Mots clés. Cilié, ultrastructure, cortex.

Summary. In this work, we compared the cortical as well as the buccal ultrastructure of the ciliated protozoans *Myxophyllum* (endocommensal of the Pulmonate) and *Myxophthirus* (endocommensal of marine Bivalves) to those observed in other ciliates such as *Conchophthirus* (endocommensal of freshwater Bivalves) and Thigmotricha species (endocommensal of marine Molluscs).
All the species studied show a locomotory cortex and a thigmotactic cortex. The locomotory cortex consists of kineties with monokinetids except *Myxophthirus* which most often shows paired and ciliated kinetosomes.
Myxophyllum presents a thin microfibrillar rope long the right side of kinetosomes whereas *Myxophthirus* has 2-3 longitudinal microtubules parallel to each kinetie as in Thigmotrichs (*Proboveria*). Ribbon of sub-kinetial microtubules are clearly seen in the cortex in *Conchophtirus*. The monokinetids of the cortical locomotory kineties have the Scuticociliate-fibrillar tangential derivates, notably the transverse tract with the ribbon of transverse microtubules.
The thigmotactic cortex always consists of dikinetids. The posterior kinetosome of the latter supports the fibrillary tangential systems similar to those associated with isolated kinetosomes. The anterior kinetosome shows some transverse microtubules and a transverse tract. The two kinetosomes of each dikinetid in *Myxophyllum*, *Myxophthirus* and *Conchophthirus* species are displaced and each kinetosome is associated to parasomal sac. In *Proboveria* the kinetosomes of each cluster are in a straight line and the middle kinetosome bears a kinetodesmal fibre similarly to the posterior kinetosome. A thigmoplasm formed by vesicles and saccules is always differentiated in the thigmotactic zone. The location of the anterior suture line of kineties which delimits both principal faces is always reinforced by a cytoskeleton consisting of microtubules into a matrix.
Both *Myxophyllum* and *Myxophthirus* endoplasm show numerous thick mitochondria whereas the chondrioma of either *Conchophthirus* or that of the Thigmotrichs (*Ancistrum*, *Proboveria* and *Boveria*) consists chiefly of long and big chondrioconts included in the ectoplasmic crests.
Overall, the paroral organelle is a stichodyade associated with a microfibrillar network.
Myxophyllum and *Myxophthirus* species have a single adoral organelle formed by several rows of kinetosomes. Among those, some support postciliary microtubular fibres. The three adoral organelles of the buccal cavity-upper limit of *Conchopthirus* differ from those observed within the Thigmotrichs. In this group indeed, the adoral organelles develop through a juxtaposition of 2-3 rows of kinetosomes-bearing postciliary microtubules.
Lastly, it seems suitable to join together both *Myxophyllum*, *Myxophthirus* from one hand and *Thigmophrya* species from the other hand within the *Thigmophryidae* group which is ranged in the Scuticociliated *Philasterina*. In addition, we propose that the *Conchopthiridae* should be distinguished into the Scuticociliates *Pleuronematina*. Since we lack ultrastructural data on the whole *Pleuronema-Histiobalantium* group, a new sub-order *Thigmotrichina* should thus be accepted.

Key words. Ciliates, ultrastructure, cortex.

INTRODUCTION

Raabe crée, en 1934, le genre *Myxophyllum*, distinct du genre *Conchophthirus* Stein, 1861, pour un Cilié présent dans le mucus et la cavité palléale de divers Pulmonés terrestres et il l'inclut, en 1963, dans la famille des *Thigmophryidae* Chatton et Lwoff, 1923. Si *Myxophyllum* steenstrupi a fait l'objet de nombreuses observations morphologiques (Rossolimo et Jakimovitsch 1929, Penn 1958, Beers 1962 a, b, Kazubski, 1973) et écologiques (Beers 1959, Kazubski 1964, Antipa et Small 1971), son ultrastructure n'a été que brièvement signalée (de Puytorac et al. 1983). Il était intéressant d'en reprendre l'étude, conjointement à

celle d'un autre Cilié, *Myxophthirus anomalocardiae* de la cavité palléale du Lamellibranche *Anomalocardia* et décrit par l'un de nous (Silva Neto 1992).

MATERIEL ET METHODES

Myxophyllum steenstrupi a été récolté en été, dans le manteau du Mollusque *Succinea putris* (L.) du Massif Central français.

Les Ciliés ont été fixés pour la technique d'imprégnation au protargol selon la technique de Bodian modifiée par Grolière (1980). Pour la microscopie électronique, les Ciliés ont été fixés au glutaraldéhyde à 2% dans du tampon cacodylate 0.05 M (pH = 7,2) pendant 15 minutes puis rincés et postfixés pendant 0 h 30 avec du tétroxyde d'osmium à 1% dans le même tampon.
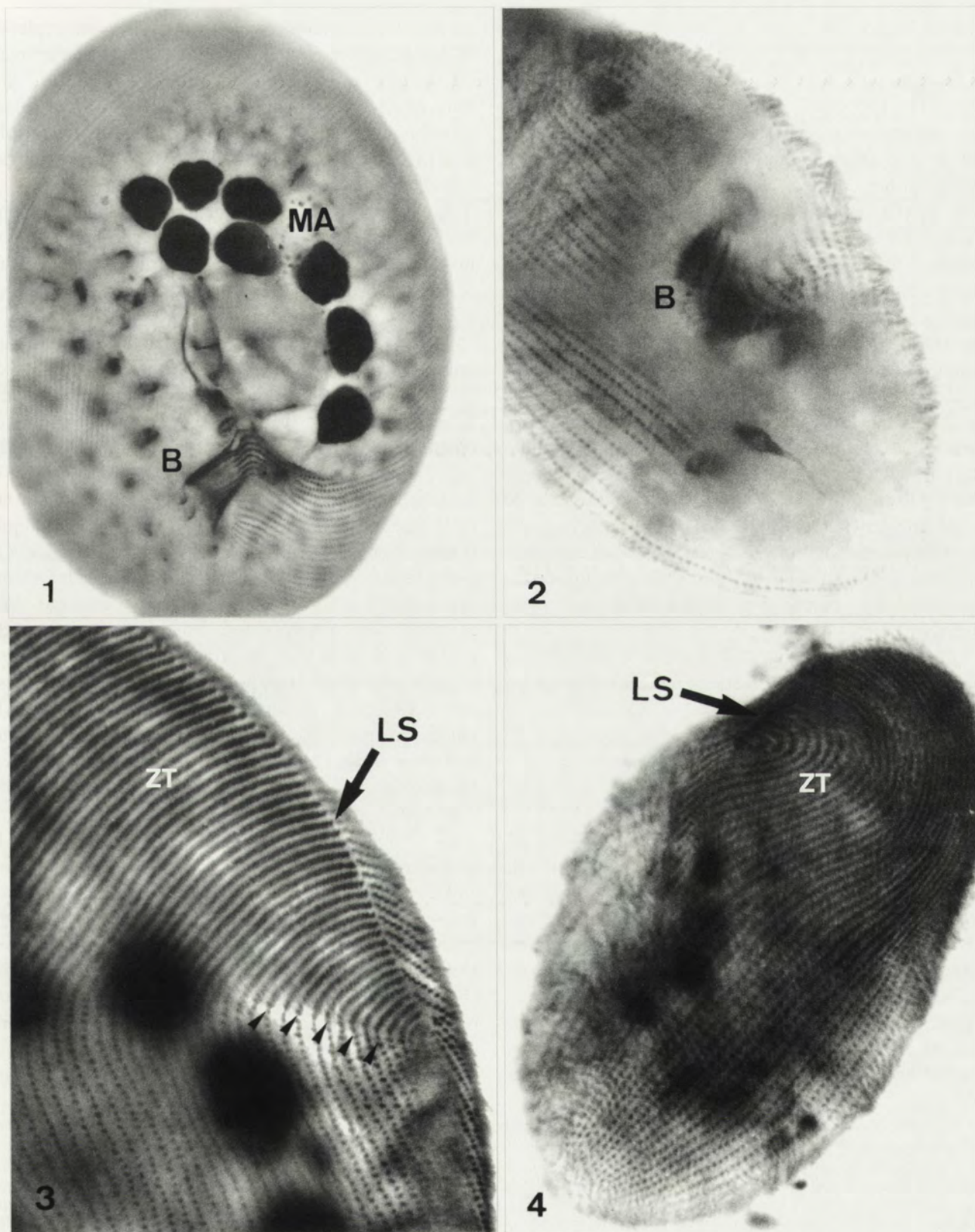
Figs 1-4. Imprégnations au Protargol de *Myxophyllum* (1,3) et de *Myxophthirus* (2,4) montrant. 1- vue générale de *Myxophyllum* avec la bouche (B) en position postérieure, et l'appareil macronucléaire (MA) (X 1000). 2- la position équatoriale de la bouche (B) de *Myxophthirus* (X 1700). 3- la ligne antérieure (LS) de suture de *Myxophyllum* avec l'aire thigmotactique (ZT) bien délimitée (flèches), aux doubles rangées de cinétosomes (X 3200). 4- la ligne de suture (LS) antérieure de *Myxophthirus* et l'aire thigmotactique (ZT) (X 1600)

RESULTATS

Morphologie. Ovoïde, aplati latéralement, le corps atteint 100-150 µm de long pour 80-100 µm de large, avec une épaisseur de l'ordre de 30 µm. La face latérale gauche est concave, thigmotactique (Fig. 3), la face latérale droite convexe. Les cinéties sont nombreuses (125), serrées, dessinant par leurs extrémités antérieures une ligne de suture séparant les 2 faces latérales de la cellule et à leurs extrémités postérieures une ligne de suture ventrale. Située dans le 1/3 postérieur du corps (Fig. 1), profondément invaginée, la cavité buccale est précédée d'un vestibule dont l'ouverture forme une ligne oblique et dont le bord droit est abrupt alors que le bord gauche est, au contraire, en pente douce, recouvert des prolongements de cinéties somatiques invaginées. L'infraciliature adorale (Fig. 5) est représentée par une plage de cinétosomes dont l'ensemble figure un triangle à base postérieure, et dans laquelle il est impossible de reconnaître les éventuelles limites de plusieurs organelles. L'organelle paroral est court et son extrémité antérieure est au niveau du milieu du champ adoral. Quelques cinétosomes isolés pourraient représenter le scuticus.

Plusieurs macronoyaux (5-7) ont une disposition en fer à cheval (Fig. 1), à l'intérieur duquel est placé un micronoyau unique.

ULTRASTRUCTURE

Ultrastructure corticale. La couche alvéolaire est généralement bien marquée, l'épiplasme mince (Fig. 7).

Les cinéties de la face latérale droite (cortex locomoteur), implantées dans des sillons délimités par de hautes crêtes ectoplasmiques (Fig. 7) dont le sommet porte un microtubule longitudinal, sont formées de monocinétides (Fig. 6).

Chaque cinétosome, bordé de part et d'autre, par des sacs parasomaux (une rangée intercalée entre les cinéties) est associé aux systèmes fibrillaires suivants:

a) une fibre cinétodesmale courte, liée au triplet 7, d'abord transversalement ou obliquement orientée avant de se diriger vers l'avant; b) un rideau de 5-6 microtubules postciliaires lié au triplet 9; c) un rideau de 6-7 microtubules transverses dans le secteur du triplet 5; d) un tractus transverse (Fig. 6) lié au secteur des triplets 4-5.

Un fin cordon microfibrillaire à noeuds, court latéralement à la droite des cinétosomes; à leur base part un faisceau microfibrillaire à structure périodique orienté transversalement à la cinétie et d'un côté, comme de l'autre, de cette dernière.

Au niveau de la zone thigmotactique de la face latérale gauche, les cinéties sont constituées de dicinétides (Fig. 9), bordées à droite comme à gauche de sacs parasomaux, de telle sorte qu'entre chaque cinétie il y a 2 rangées de sacs parasomaux (Fig. 11). Les 2 cinétosomes d'une dicinétide sont cilifères et le cinétosome postérieur est légèrement décalé sur la droite de la cinétie par rapport au cinétosome antérieur auquel il est relié par une desmose oblique bien marquée. On retrouve au niveau du cinétosome postérieur les même systèmes fibrillaires tangentiels que ceux qui sont associés aux cinétosomes isolés. Au niveau du cinétosome antérieur, un tractus transverse et 1-2 microtubules transverses peuvent être présents. A la base des cinétosomes postérieurs court parallèlement à la cinétie un ruban microfibriliaire à structure périodique de bandes épaisses contrastées, alternant avec des interbandes plus étroites (Fig. 10). De ces mêmes cinétosomes partent de courts faisceaux microfibrillaires transversaux dirigés vers la droite jusqu'au niveau de la cinétie voisine (Fig. 10).

Des chondriocontes sont présents au niveau des crêtes ectoplasmiques mais de nombreuses mitochondries dont certaines cupuliformes, enfermant une Bactérie non envacuolée, sont aussi dispersées dans l'endoplasme (Fig. 7). Ce dernier est très riche en réticulum endoplasmique et en Bactéries envacuolées, en vacuoles alimentaires. Des saccules et vésicules de type Golgien sont abondants dans le cytoplasme du cortex thigmotactique,

Fig. 5. Imprégnation au Protargol de *Myxophyllum* montrant l'aire buccale avec les cinéties vestibulaires (CV), l'organelle paroral (PA) et l'infraciliature adorale (OA) (X 1150)

Figs 6-8. Electronographies de *Myxophyllum* (6,7) et de *Myxophthirus* (8). 6- Coupe tangentielle du cortex locomoteur de *Myxophyllum* montrant les monocinétides avec fibre cinétodesmale (KD), rideau de microtubules postciliaires (PC), tractus transverse (TT), rideau de microtubules transverses (T), sacs parasomaux (SP), épiplasme (EP), mitochondries (M) (X 152000). 7- Coupe transversale de *Myxophyllum* au niveau du cortex locomoteur montrant les cinétosomes implantés dans les sillons délimités par les crêtes ectoplasmiques (CR), les alvéoles corticales (AL), l'épiplasme (EP), les mitochondries (M) corticales et endoplasmiques, des amas de vésicules golgiennes (SG), des Bactéries (BA), des tractus microfibrillaires, à structure périodique (RF) (X 20000). 8- Coupe tangentielle du cortex locomoteur de *Myxophthirus* montrant les dicinétides avec, au niveau du cinétosome postérieur: fibre cinétodesmale (KD), rideau de microtubules postciliaires (PC), tractus transverse (TT), microtubules (MT) courant parallèlement aux cinéties, sacs parasomaux (SP), couche alvéolaire (AL) mitochondrie (M) (X 18000)
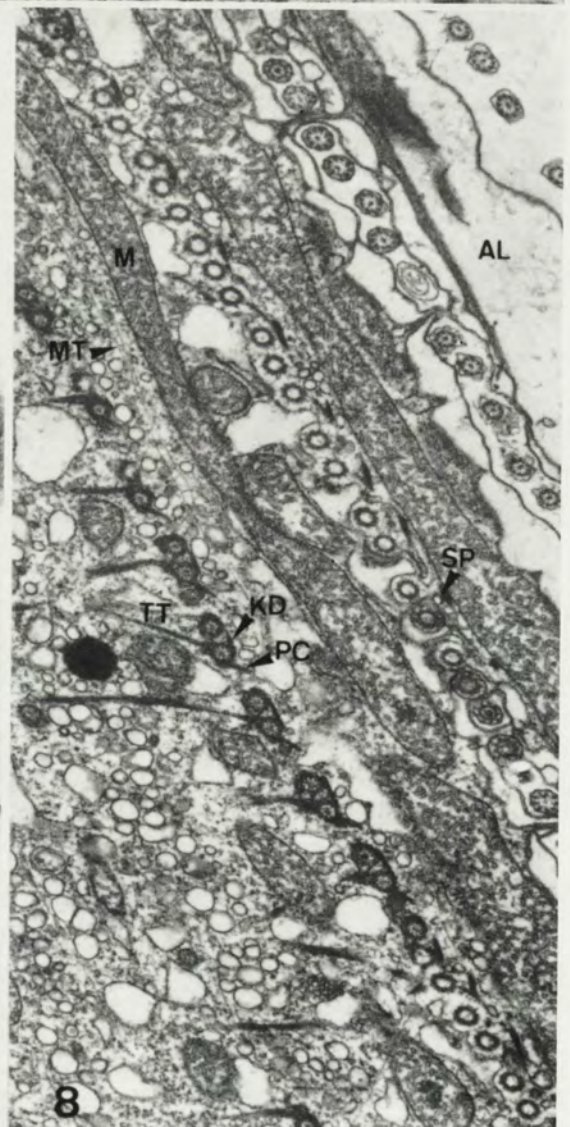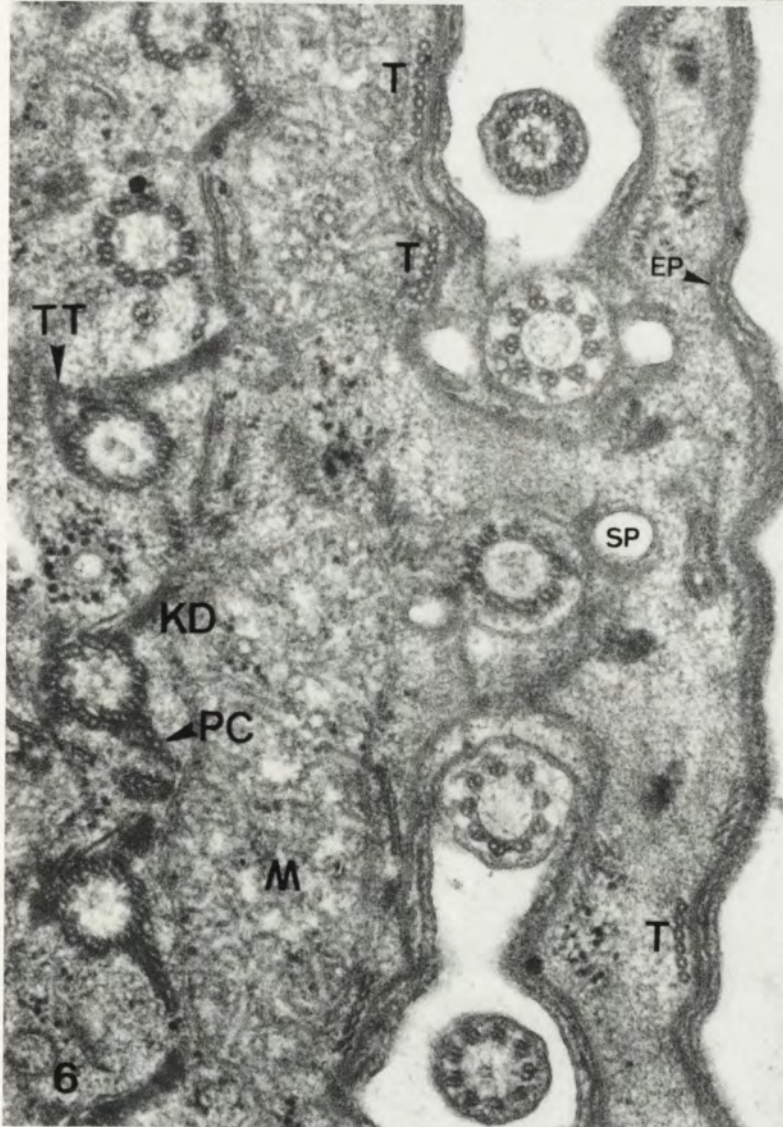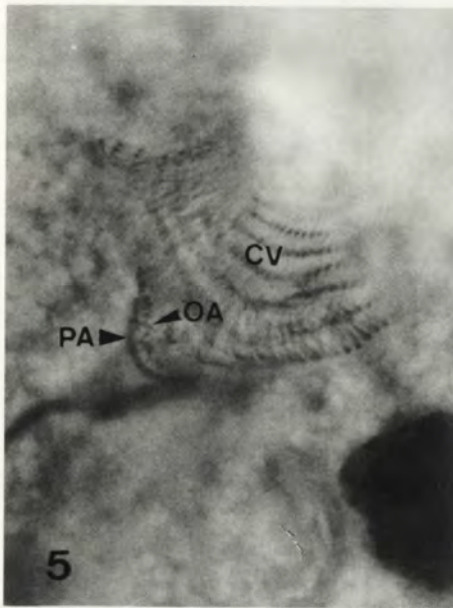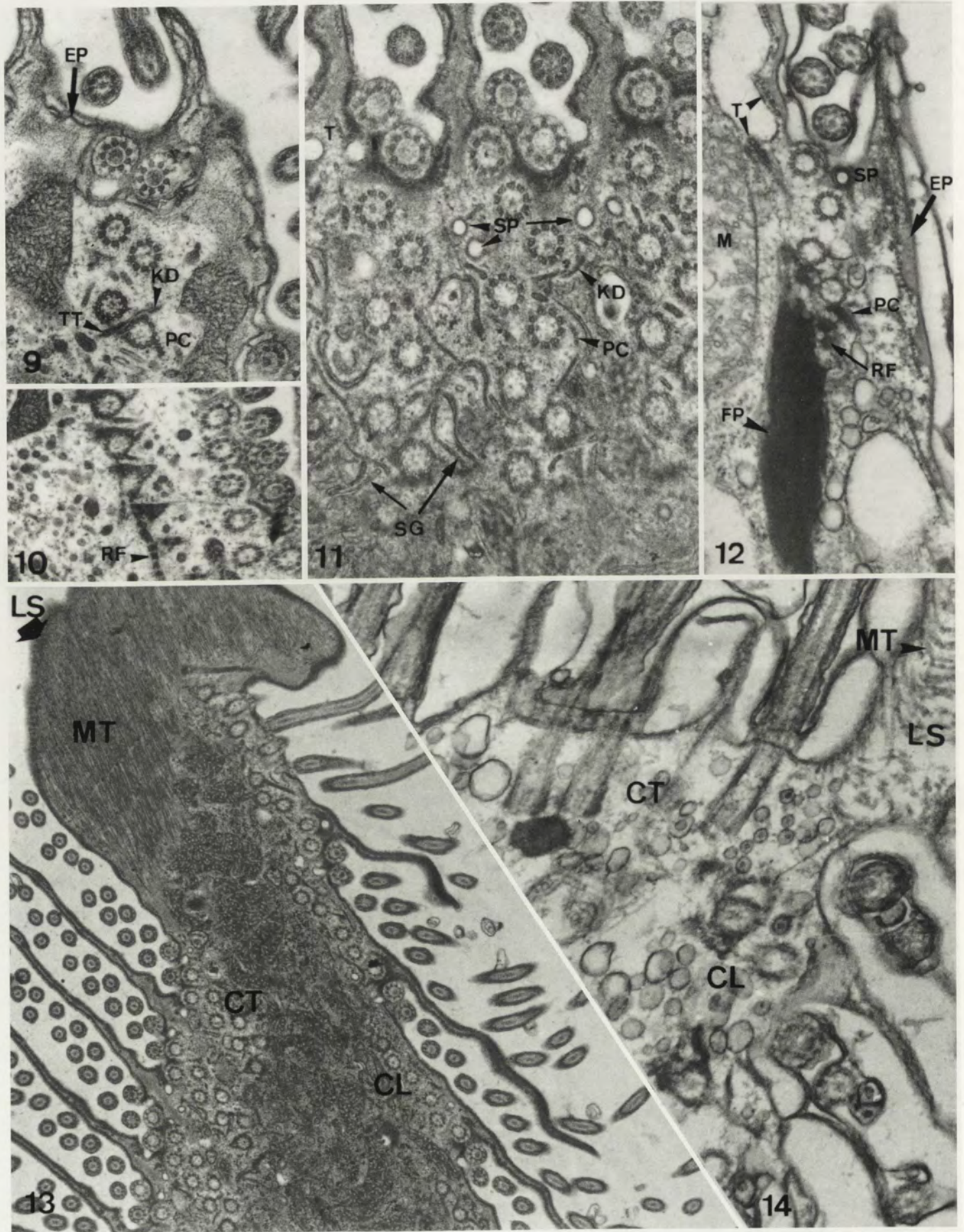
participant à la constitution du thigmoplasme (Fig. 11). Des vésicules à contenu granuleux, filamenteux ou tubulaires sont nombreuses dans la partie postérieure de la cellule (Fig. 15). La ligne antérieure de suture est occupée par un amas dense de microtubules parallèlement alignés, dans une matrice (Fig. 13).

Ultrastructure buccale. L'organelle paroral (Figs. 5, 15, 17) est une stychodyade dont les cinétosomes ciiiés de la rangée externe sont bordés de sacs parasomaux et dont les cinétosomes nus de la rangée interne sont pourvus d'un rideau de microtubules postciliaires. Un réseau microfibrillaire à noeuds de condensation est sous-jacent à la parorale en s'étalant dans la zone des crêtes orales (Figs. 16, 17). Chaque crête supporte 2 rangées inégales de microtubuies (7 + 3).

Le champ adoral est formé de rangées de cinétosomes dont certains portent des fibres microtubulaires postciliaires (Fig. 16).

DISCUSSION

Comparaison avec l'ultrastructure de *Myxophthirus anomalocardiae* Da Silva Neto, 1992.

Rappelons que chez ce Cilié d'un Lamellibranche du Brésil (*Anomalocardia brasiliana*), la région antérieure de la face gauche est déprimée en ventouse (Fig. 4) et que la cavité buccale, précédée d'un vestibule, est immédiatement sous-équatoriale (Fig. 2). L'infraciliature buccale est formée d'une parorale courte et d'un champ adoral d'une dizaine de rangées de cinétosomes.

L'ultrastructure de *Myxophthirus* est du même type que celle de *Myxophyllum* tant au niveau cortical que buccal. Cependant, au niveau du cortex locomoteur de *Myxophthirus* (Fig. 8), les cinéties sont, le plus souvent, formées de dicinétides, aux deux cinétosomes cilifères et chez *Myxophyllum* sont absents les 2-3 microtubules longitudinaux parallèles à chaque cinétie présents à la base des cinétosomes et à leur droite chez *Myxophthirus*

(Fig. 8); ils sont remplacés chez *Myxophyllum* par un fin cordon microfibrillaire à noeud. Au niveau du cortex thigmotactique (Figs.12, 14), les cinéties sont à dicinétides avec les mêmes dérivés fibrillaires tangentiels que chez *Myxophyllum*. Cependant, dans le cortex thigmotactique de *Myxophthirus* une fibre périodique squelettique, accolée aux extrémités proximales des cinétosomes (Fig. 14), longeant la gauche de chaque cinétie, renforce la ventouse (Fig. 12).

Tant chez *Myxophyllum* que *Myxophthirus*, l'organelle paroral en stichodyade est associé à un réseau microfibrillaire à noeuds et certains cinétosomes de l'organelle adoral portent de longs rideaux de fibres postciliaires.

Il est donc justifié de réunir les genres *Myxophyllum* et *Myxophthirus* dans une même famille avec *Thigmophrya* Chatton et Lwoff, 1923, genre de Ciliés parasites de la cavité palléale de Bivalves marins et dont la cavité buccale est postérieure, précédée d'un vestibule à parois dissymétriques et dont l'infraciliature adorale serait aussi sous forme d'un champ cinétosomien (Fenchel 1964).

La rétrogradation buccale est moins avancée chez *Myxophthirus* que chez *Myxophyllum* et *Thigmophrya*. L'inclusion dans cette famille des *Thigmophryidae* du genre *Conchophyllum* créé par Raabe (1936) pour l'espèce *C. caryoclada* Kidder, 1933, commensal du Bivalve *Siliqua patula* à cavité buccale située dans le 1/4 postérieur de la cellule, à ciliature buccale inconnue, est moins évidente. Il en est de même pour le genre *Cochliophilus* Kozloff, 1945 commensal de Pulmonés (*Phytia*) dont la cavité buccale postérieure n'est pas précédée d'un vestibule et dont l'infraciliature buccale est inconnue et pour le genre *Cochliodomus* Raabe, 1971 dont l'espèce *C. oncomelaniae* (Tchang et al. 1957) de la cavité palléale de Gastropodes est totalement à redécrire.

Raabe place aussi dans la famille des *Thigmophryidae*, outre la sous-famille des *Thigmophryinae*, les sous-familles des *Conchopththirinae* et des *Peniculistomatidae*.

Figs 9-14. Electronographies de cortex de *Myxophyllum* (9, 10, 11, 13) et de *Myxophthirus* (12, 14). 9, 10, 11- Coupes tangentielles du cortex thigmotactique de *Myxophyllum* montrant les dicinétides, avec sur le cinétosome postérieur la fibre cinétodesmale (KD), le rideau de microtubules postciliaires (PC), le tractus transverse (TT); les sacs parasomaux (SP), les tractus microfilamenteux (RF) à structure périodique sous-cinétiens, les saccules de type golgien (SG) du thigmoplasme l' épiplasme (EP) (9: X 30000; 10: X 24000; 11: X 40000). 12- Coupe tangentielle du cortex thigmotactique de *Myxophthirus* montrant avec, sur le cinétosome postérieur, le rideau de microtubules postciliaires (PC), des rideaux de fibres transverses (T); les sacs parasomaux (SP), la fibre squelettique sous-cinétienne (FP) longeant la gauche des cinéties, l'épiplasme (EP) localement épaissi une mitochondrie (M) (X 29000). 13- Coupe de *Myxophyllum* passant par la ligne de suture antérieure (LS) avec la masse sous-jacente des microtubules (MT) et les cortex locomoteur (CL) à monocinétides et thigmotactique (CT) à dicinétides (X 16000). 14- Coupe passant par la ligne de suture antérieure (LS) de *Myxophthirus* avec les microtubules (MT), le cortex locomoteur (CL) et thigmotactique (CT), (X 45000), la position de la fibre squelettique (FP) sous-cinétienne (X 45000)

Comparaison de l'ultrastructure des genres *Myxophyllum*, *Myxophthirus* avec celle du genre *Conchophthirus* Stein, 1861 dont les espèces sont parasites de Mollusques Bivalves d'eau douce (*Unio*, *Anodonta*) (Kidder 1933 a, b, c, 1934; Penn 1958; Beers 1959; Raabe 1936, 1947, 1963).

L'ultrastructure corticale des *Thigmophryidae* a des similitude avec celle de *Conchophthirus* (Antipa 1971); même type de cortex locomoteur à monocinétides (chez *Myxophyllum*), de cortex thigmotactique à dicinétides; mêmes types de dérivés fibrillaires tangentiels. Cependant, des microtubules sous-cinétosomiens ne sont présents que chez *Conchophthirus*. Quelques microtubules longent la base des cinétosomes de *Myxophthirus*. Ils manquent chez *Myxophyllum*. En outre, le chondriome de *Conchophthirus* est essentiellement à base de longs chondriocontes inclus dans les crêtes ectoplasmiques, alors que dans les 2 autres genres, les mitochondries, de formes variées, dispersées dans le cytoplasme sont une fraction plus importante que les mitochondries périphériques et il n'y a pas de cristallocystes, mais des mucocystes et/ou des inclusions de types divers. Dans les 3 genres, la zone de la ligne de suture antérieure est renforcée par un cytosquelette de microtubules noyés dans une matrice plus ou moins dense.

L'organisation buccale de *Conchophthirus* est très différente de celle des *Thigmophryidae*. L'appareil buccal de *Conchophthirus* est situé dans le 1/4 antérieur du corps et ce sont quelques cinéties droites qui représentent des cinéties vestibulaires alors que chez les *Thigmophryidae*, ce sont des cinéties gauches qui s'invaginent dans le vestibule.

En outre, chez *Conchophthirus*, il y a 3 organelles adoraux au plafond de la cavité buccale, parallèlement alignés en se chevauchant un peu, formé chacun de 3 rangées de cinétosomes, au lieu d'un champ adoral des *Thigmophryidae*. Enfin, l'organelle paroral est long, chez *Conchophthirus*, doublé par une scuticocinétie alors qu'il est court et très réduit chez les *Thigmophryidae*. Il semble donc justifié de distinguer une famille des *Conchophthiridae* Kahl in Doflein et

Reichenow, 1929 et une famille des *Thigmophryidae* Chatton et Lwoff, 1923.

La première est considérée par Corliss (1979), Small et Lynn (1985) comme appartenant aux Scuticociliés *Pleuronematina*, la deuxième aux Scuticociliés *Philasterina*. Chez ces derniers: a) les primordiums oraux dérivent de champs cinétosomiens développés les uns à partir de la parorale, les autres à partir du scuticus, et cela selon des types différents (Grolière 1974, 1980) correspondant aux différences de potentialités morphogénétiques respectives de la parorale et du scuticus; b) la structure buccale parentale se dédifférencie peu, au cours de la division cellulaire. Chez les *Pleuronematina*: a) les primordiums oraux dérivent de la seule parorale (*Pleuronema*, Grolière et Detcheva, 1974; *Histiobalantium*, *Peniculostoma*, *Mytilophilus* Dolan et Antipa, 1965) ou dans le cas de *Conchophthirus* (Antipa et Hatzidimitriou, 1981) comme de *Cyclidium* (Grolière, 1980); *Ancitrum* (Hatzidimitriou et Berger, 1971) de la parorale et d'un scuticus; b) la structure buccale parentale subit une importante dédifférenciation puis redifférenciation.

En l'absence de données convenables sur la stomatogénèse des *Thigmophryidae*, l'éloignement de cette famille avec celle des *Conchophthiridae* peut être maintenue.

Comparaison de l'ultrastructure des genres *Myxophyllum*, *Myxophthirus* avec celle des Thigmotriches.

Chez *Ancistrum*, *Boveria* (Lom et all., 1968), *Proboveria* (de Puytorac et coll., 1978) comme chez les *Thigmophryidae*, la couche alvéolaire est bien marquée et l'épiplasme est présent, mais ce sont là des caractères généraux aux Scuticociliés, Péniculiens, Péritriches. Les zones ciliaires locomotrices sont à monocinétides, les zones thigmotactiques à di- ou tricinétides mais alors que chez *Myxophyllum* et *Myxophthirus* (comme chez *Conchophthirus*), les 2 cinétosomes des dicinétides sont décalés l'un par rapport à l'autre et accompagné chacun d'un sac parasomal, chez *Proboveria* les cinétosomes

Figs 15-18: Electronographies d'aires buccales de *Myxophyllum* (15-17) et de *Myxophthirus* (18). 15- Coupe au niveau de la cavité buccale, au fond de l'infundibulum (INF) montrant la gouttière contenant la parorale (PA), les crêtes orales (CR), la concavité contenant l'infraciliature adorale (AD), des vésicules de contenu fibreux (VF) (X 20000). 16- Coupe tangentielle de l'infraciliature adorale (AD) montrant des rideaux de fibres postciliaires (PC) portés par des cinétosomes de différentes rangées et un réticulum microfilamentaire à noeuds (RF) à la base des cinétosomes (X 32000). 17- Coupe passant par l'organelle paroral (PA) montrant la rangée externe de cinétosomes ciliés bordée par les sacs parasomaux (SP), la rangée interne de cinétosomes nus porteurs de microtubules postciliaires (PC), le réseau à noeuds de condensation (RF), les crêtes orales (CR) (X 37000). 18- Coupe de *Myxophthirus* au niveau de l'organelle paroral (PA) inclus dans une gouttière délimitée par des crêtes (CR) avec couche alvéolaire (AL) et de l'infraciliature adorale. Microtubules postciliaires (PC). (X 22000)
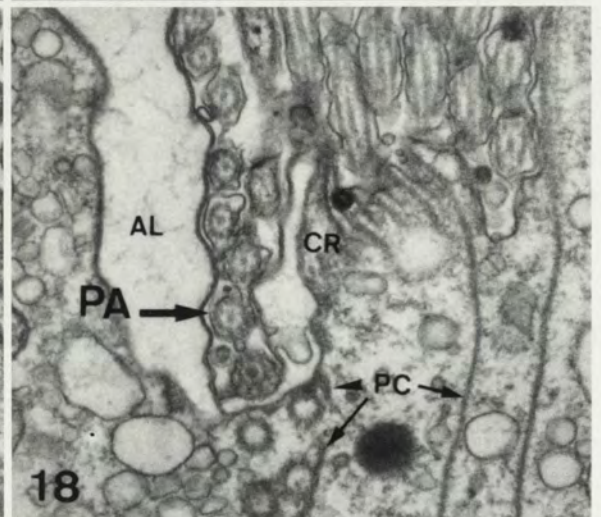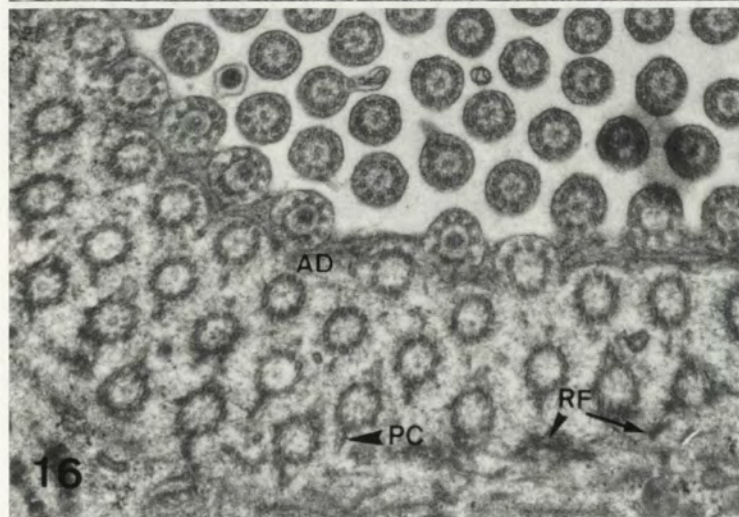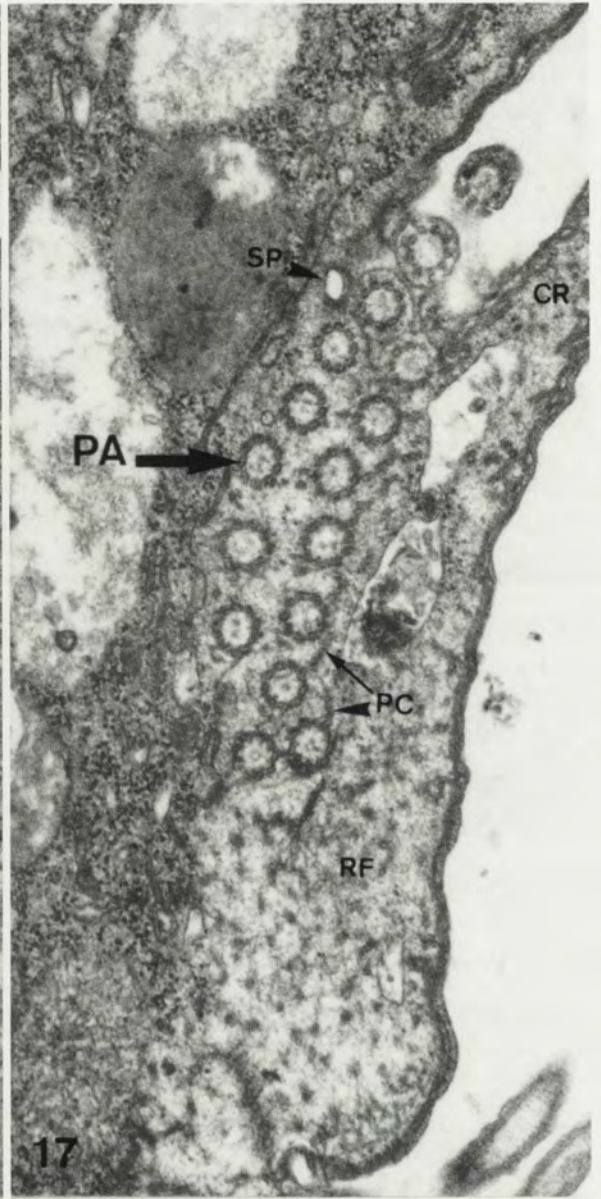
groupés sont en file, avec un seul sac parasomal médian, à droite.

Chez *Proboveria*, comme chez *Myxophyllum*, *Myxophthirus* (et *Conchophthirus*) au niveau des cinétosomes isolés ou au niveau du cinétosome postérieur (dans les groupes de 2-3 cinétosomes), un tractus transverse est présent, outre le rideau des microtubules transverses, mais c'est aussi le cas pour les Scuticociliés et les Tétrahyméniens. Chez *Proboveria*, par contre, une fibre cinétodesmale courte est portée par le cinétosome moyen et/ou antérieur, s'ajoutant à la fibre cinétodesmale un peu plus longue du cinétosome postérieur. Cet état ne se retrouve pas chez les *Thigmophryidae* et les Scuticociliés. Des microtubules longitudinaux accompagnent les cinéties, à la base ou au côté des cinétosomes tant chez *Proboveria* que *Myxophthirus* (et *Conchophthirus*).

Un thigmoplasme à base de saccules et de vésicules est toujours différencié au niveau de la zone thigmotactique. Chez *Proboveria*, comme chez les *Thigmophryidae* (et *Conchophthirus*), l'emplacement de la ligne antérieure de suture des cinéties est garni de microtubules parallèlement alignés. Le chondriome à chondriocontes intercinétiens des *Ancistrum*, *Boveria*, *Proboveria* est plus proche de celui de *Conchophthirus* que du chondriome à mitochondries éparses des *Thigmophryidae*.

Dans tous les cas, l'organelle paroral est une stychodyade comme chez les Scuticociliés, Tétrahyméniens, Péniculiens, Péritriches. Les 3 organelles adoraux d'*Ancistrum*, *Boveria*, *Proboveria* sont constitués de la juxtaposition de 2 ou 3 rangées de cinétosomes dont chacune porte des rideaux de microtubules postciliaires, ce qui n'est pas le cas pour l'organelle adoral des *Thigmophryidae*, où ne se retrouvent pas, en outre, les desmoses épaisses reliant les bases cinétosomiennes comme dans certains membranoïdes des Scuticociliés (C. Bouland et coll., 1986-87).

Bien que partageant avec les Thigmotriches des caractères ultrastructuraux communs aux Scuticociliés, les *Thigmophryidae* ont donc des caractéristiques différentes de celles des Thigmotriches.

Pour ces derniers, Corliss (1979) puis Small et Lynn (1985), de Puytorac et al. (1987) maintiennent dans les *Scuticociliatida* Small, 1967 un sous-ordre des *Thigmotrichina* Chatton et Lwoff, 1922, avec les familles des *Ancistridae* Issel, 1903; *Hemispeiridae* König, 1894; *Hysterocinetidae* Diesing, 1866; *Protoanoplophryidae* Miyashita, 1929; *Nucleocorbulidae* Santhakumari et Nair, 1970.

Mais depuis les observations de Ngassam et col. (inédit), on sait que les *Hysterocinetidae*, tant par leur ultrastructure que leurs processus stomato-génétiques sont bien distincts des Thigmotriches.

Chez *Ancistrum mytili*, le scuticus est formé de 2 segments de cinétosomes nus, alignés parallèlement à la partie postérieure de la parorale et au dessous d'elle, ce qui n'est pas sans évoquer la scuticocinétie de *Conchophthirus*.

Par la stomatogénèse, les *Ancistridae* diffèrent nettement des *Peniculostomatidae* et *Histiobalantiidae*. On peu alors se demander si les *Thigmotrichina*, même épuré des *Hysterocinetidae*, représente un ensemble monophylétique et si ce sous-ordre doit être maintenu tel quel avec celui des actuels *Pleuronematina*. En l'absence de données ultrastructurales sur l'ensemble *Pleuronema-Histiobalantium*, on peut accepter un sous-ordre des *Thigmotrichina*, avec un sous-ordre des *Pleuronematina*, les *Thigmophryidae* appartenant en tous cas au sous-ordre des *Philasterina*.

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ACTA PROTOZOOLOGICA

The Morphology and Ultrastructure of the Antarctic Ciliate, *Cymatocylis convallaria* (Tintinnina)

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Summary. The morphology and ultrastructure of the tintinnid ciliate *Cymatocylis convallaria*, a planktonic species restricted to Antarctic waters, were examined. The structures common for all ciliates, as well as those characteristic of tintinnids, are described. The ultrastructure of the lorica changes from trilaminar at the horne, through bilaminar in the middle part, to monolaminar near the collar. The capsules are typical intracellular elements for all tintinnids, however, fibrillar connections between them and the affiliated caps as well as between the caps and plasmalemma are described for the first time. The structures called "morulas", formed by loosely and closely packed osmophilic globules, are noticed. The simultaneous presence of differently compact "morulas" was not previously reported. Although the ultrastructure of *C. convallaria* is typical for Tintinnina in general, some features seem to be characteristic for this species.

Key words. lorica, ciliature, perilemma, intracellular organelles.

INTRODUCTION

The cells belonging to suborder Tintinnina are an abundant group among the Antarctic marine protozooplankton (Boltovskoy et al. 1989, Brandini and Kutner 1987, Garrison and Buck 1989). Studies on morphology and physiology of the cells belonging to this suborder began at the end of the ninetheenth century (Entz 1885, Daday 1887, Hofker 1931). Afterwards precise descriptions of some genera were made by Campbell (1926) and Biernacka (1952).

The studies of the ultrastructure of tintinnids were initiated by Laval (1971) and have been continued

until the present (Hedin 1975, 1976b, Laval 1972, Laval-Peuto 1975, 1980, Laval-Peuto and Brownlee 1986, Laval-Peuto et al. 1979, Sokolova and Gerassimova 1984, Sokolova et al. 1986, Wasik and Mikołajczyk 1991). Additional information about the infraciliature has been obtained by protargol-silver staining (Brownlee 1977, Choi et al. 1992, Foissner and Wilbert 1979, Hedin 1976a, Laval-Peuto and Brownlee 1986).

In spite of the expansion of studies on tintinnids very little is known about the ultrastructure of the species restricted to Antarctic waters. Therefore, the purpose of our study was to describe the ultrastructure of *Cymatocylis convallaria*, which is one of the most abundant species in this region (Alder and Boltovskoy 1991, Boltovskoy et al. 1989, Wasik and Mikołajczyk 1990).

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MATERIAL AND METHODS

Cymatocylis convallaria (Laackmann, 1909) cells were collected in Admiralty Bay of the King George Island and near the South Shetland Islands in January 1989 during an Antarctic expedition organized by the Institute of Ecology the Polish Academy of Sciences. The cells were fixed at 0-3°C immediately after collection in: precooled 4% formalin buffered with calcium carbonate, 1% osmium tetroxide with sublimate (1:1) or 2.5% glutaraldehyde. The fixed cells were transported to the laboratory at the Nencki Institute of Experimental Biology where they were further processed and examined.

For scanning and transmission electron microscopy cells were prepared as previously described (Wasik and Mikołajczyk 1991).

The protargol-silver staining was carried out according to Hedin (1976 a) with minor modifications: impregnation in protargol (Roques) was for 30 min. at 60°C.

The cells were viewed on a JEOL 1200 EX electron microscope equipped with an ASID 19 scanning attachment operating at 40 kV and on a JEOL 1200 EX transmission electron microscope operating at 80 kV. The material was also examined under light microscope (Leitz and Jenalumar, Carl Zeiss, Jena).

RESULTS

Cymatocylis convallaria is protected by a bell shaped, smooth, hyaline lorica ending in a small, blunt horn (Fig. 1). The total length of the lorica is about 110 μm, while the oral diameter is 90 μm. The collar is well developed, with a striated oral rim and a denticulated inner erected crest (Fig. 1). The ultrastructure of the lorica is rather complex. It is trilaminar in the horn zone (Fig. 2); the external and internal layers have the same thickness (ca 100 nm) and are divided into almost rectangular compartments; the middle layer, which is formed by netted structures, can reach a thickness of 500 nm and sometimes even more. Towards the oral margin, the middle layer gradually declines and the lorica becomes bilaminar (Fig. 3) turning finally into monolaminar near the collar. The monolaminar part of the lorica periodically narrows, forming knots (Fig. 4). The collar is constructed of an irregular net (Fig. 5).

The cell body length is about 70-90 μm. It is measured as a distance from the base of the adoral membranelles to the aboral end, which is attached to the lorica by an adhesive disc. The oral diameter is about 40 μm. The cell body length can change since the cell is able to contract rapidly. The contractile zone is located in the *C. convallaria* aboral end (Fig. 6). In the contracted stage, this zone is darker and therefore easily distinguished from the rest of the cytoplasm. In this region there are a lot of deep surface invaginations
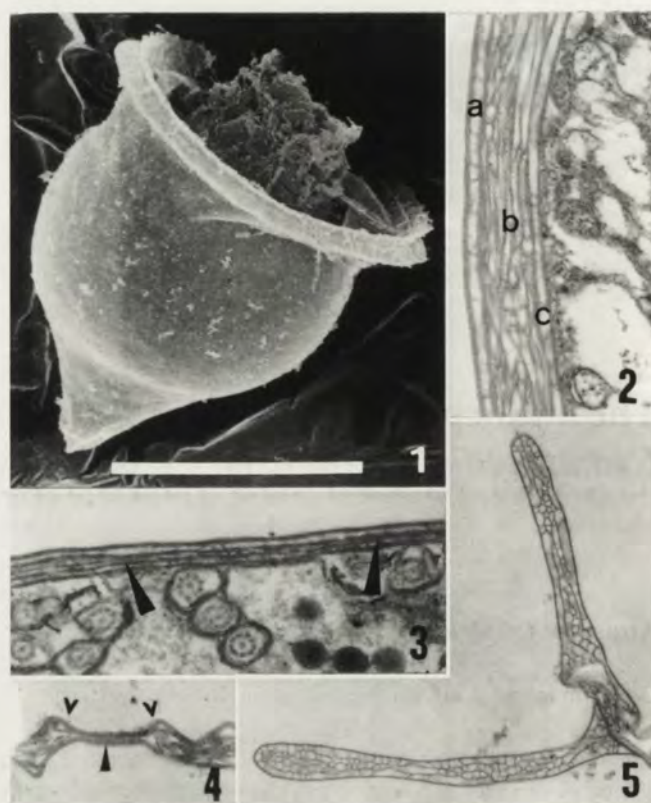
Fig. 1. Overview of *Cymatocylis convallaria*. SEM. Bar = 100 μm

Figs. 2-5. Ultrastructure of the lorica. TEM. 2. Trilaminar lorica. a - external, b - middle, c - internal layers. x 20,000. 3. Transition region from trilaminar to bilaminar lorica (arrowheads). x 10,000. 4. Monolaminar lorica (full arrowhead) with knots (open arrowheads). x 12,000. 5. The collar. x 10,000

(Fig. 6). In the state of relaxation these invaginations flatten and the aboral end becomes elongated into a slender peduncle.

The cytostome-cytopharynx complex extends posteriorly and almost reaches the contractile zone. The peristome is surrounded by 19 adoral membranelles (AZM) comprising three rows of almost equal 20-30 μm long cilia (Fig. 7). The proximal ends of the membranelles form a sinistral spiral. The kinetosomes of the membranelles are linked by a filamentous net (Fig. 8). The kinetosomes in each column are connected by transversal filaments, while the kinetosomes in the rows are linked by anterior, median and posterior connections. Additionally, diagonal fibrillar connections link the kinetosomes of the neighbouring columns. From the rows of the marginal kinetosomes, lateral microtubules extend; transversal microtubules have not been discerned.

Three striae, bulges of the membrane, are connected to each adoral membranelle (Fig. 9). Usually two of
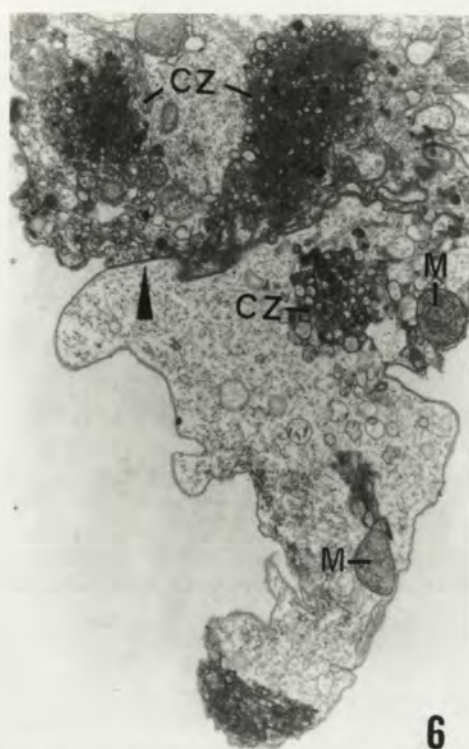
Fig. 6. Ultrastructure of the aboral end with the contractile zone (CZ). M - mitochondria, arrowhead - surface invagination. TEM. x 5,000

them are located on the internal, while one on the external side of the membranelle. Between the membranelles, small cytoplasmic extensions, called accessory combs, are located (Fig. 10). The paroral membrane is composed of a single row of cilia (Fig. 11), whose kinetosomes are connected to each other by triple fibrillar bridges. At the distal ends of the adoral and paroral cilia, swellings of the ciliary membrane are visible (Fig. 10). These modified cilia are termed "paddle cilia" or "discocilia", and have been previously described in *C. convallaria* by Wasik and Mikołajczyk (1991).

The somatic ciliature in *C. convallaria*, as in many tintinnids, is reduced. It consists of one ventral (Fig. 12) and one dorsal kinety, which begin near the AZM and run posteriorly to the aboral end. The somatic ciliature is made up of left and right ciliary fields. A posterior kinety, composed of widely spaced kinetosomes, is located on the posterior part of the cell body. Single cilia are irregulary dispersed on the cell surface. The cell body of *C. convallaria* is covered by plasmalemma and additionaly by perilemma (Figs. 9, 13). Both of them surround the entire cell body including invaginations located in the contractile zone, adoral and paroral membranelles (with their blebs,

accessory combs and striae) as well as the somatic cilia.

Very interesting structures called capsules are observed within the striae (Fig. 9) and accessory combs (Fig. 10), and also in the cytoplasm of the posterior part of the cell. They are usually positioned close to the plasmalemma (Fig. 13). The capsules are slightly oval (ca 230 x 210 nm), and covered by a unit membrane. Inside the capsule, an electron-dense truncated cone, formed by laminated layers, is visible (Fig. 13). This structure is recognized on the cross section as a ring. Beneath the cone there is a lighter striated region (Fig. 13). The anterior pole of the capsule can be distinguished from the posterior one by the presence of a cap, situated over the capsule. This cap is connected with the capsule by fibrilles. The cap of each capsule adjacent to the plasmalemma is also connected to it by fibrilles. Very often the capsules are accompanied by mucocyst-like structures.

The structures called "morulas", which are surrounded by the unit membrane, are spherical or slightly ovoid. They can vary in size, from very small to large (4 µm). They are formed by osmophilic globules from loosely to closely packed (Fig. 14). Their localization depends on the stage of the cell life cycle. In the interphase they are observed in the posterior part of the cell body. During division they move toward the anterior end of the cell, to be finally located in the daughter cell, where they are believed to take part in the process of lorica formation.

The nuclear apparatus in *C. convallaria* is composed of two macro- and two micronuclei. Inside the macronucleus numerous nucleoli are located.

In the cytoplasm numerous spherical mitochondria with tubular cristae are observed (Fig. 6). Most of them are located near the anterior and posterior parts of the cell. Close to the mitochondria, smooth and rough endoplasmic reticulum are present.

DISCUSSION

Three features are basis to the individualization of the suborder Tintinnina from the phylum Ciliophora: (1) the presence of the lorica, (2) the presence of the adoral membranelles [AZM] in an anterior crown, (3) the reduction of the somatic cilia.

For many years the morphology of the lorica was the main taxonomic distinction. However, as it was shown by some authors (Laval-Peuto 1981, Laval-Peuto and
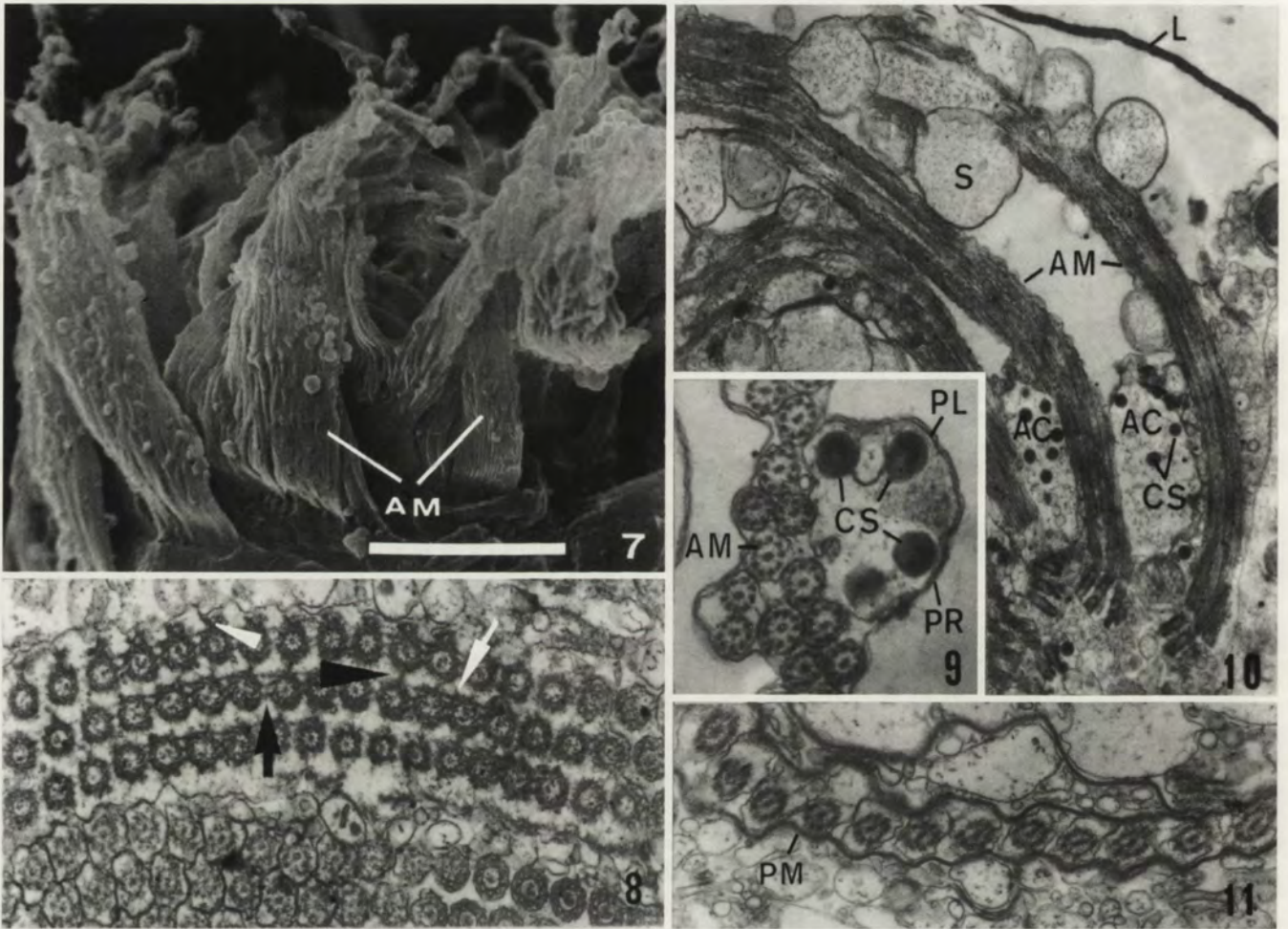
Fig. 7. Adoral membranelles (AM). SEM. Bar = 100 μm.

Fig. 8. Kinetosomes of adoral membranelle, white arrow - diagonal connections, white arrowhead - lateral microtubules, black arrow - anterior, median and posterior connections, black arrowhead - transversal connections. TEM. x 20,000.

Fig. 9. Stria on the adoral membranelle (AM). CS - capsules, PL -plasmalemma, PR - perilemma. TEM. x 20,000.

Fig. 10. Adoral membranelles (AM) with accompanied structures. L - lorica, AC - accessory comb, S - ciliary swelling, CS - capsules. TEM. x 7,000

Fig. 11. Paroral membranelle (PM). TEM. x 20,000

Brownlee 1986), this system has led to erroneous and artificial decisions. It should be emphasized that even within the same species variability in the lorica shape and size is observed (Boltovskoy et al. 1990, Laval-Peuto 1976, 1977). It has appeared that also the ultrastructure of the lorica is different depending on the species (Laval-Peuto 1980). For example, the lorica is monolaminar in *Parafavella gigantea* and *Favella ehrenbergii* (Hedin 1975), while is trilaminar in *Petalotricha ampulla* (Laval 1972). In *C. convallaria* the ultrastructure of the lorica is different to that of other tintinnids, because it changes from threelaminar at the horn, through bilaminar in the middle part, to monolaminar near the collar.

The somatic ciliature of *C. convallaria*, like in other tintinnids, is reduced and no longer participates in swimming. The locomotion functions are taken over by the oral ciliature exclusively. It has been suggested (Brownlee 1977) that the somatic cilia may be involved in lorica formation, in the positioning and maintenance of the cell body within the lorica and in removing waste from the lorica. According to Laval-Peuto and Brownlee (1986) the degree of the reduction of the somatic ciliature and the degree of their specialization points to their stage of evolution. The Tintinnina with many long kineties per cell circumference, low specialization of somatic cilia, and the strictly anterior position of the oral apparatus would be considered the
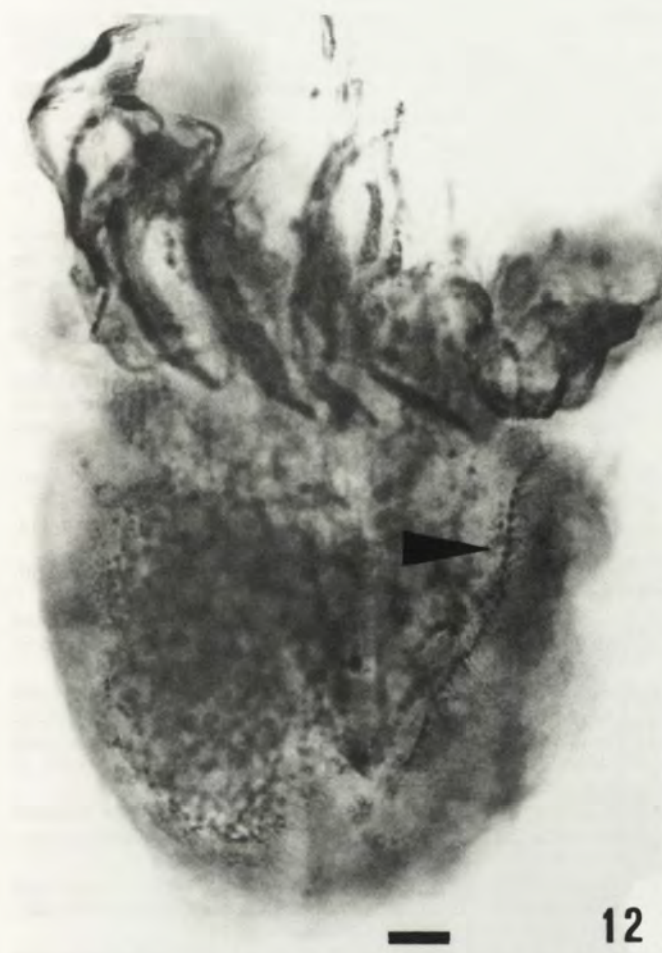
Fig. 12. Protargol impregnation of *C. convallaria*. arrowhead - ventral kinety. LF. Bar = 10 μm

Fig. 13. Capsules located in the stria. black C - cap, white C - truncated cone, SR - striated region, PL - plasmalemma, PR - perilemma. TEM. x 60,000

Fig. 14. "Morulas". a - loosely packed, b - transitional form, c - closely packed. TEM. x 15,000

most primitive, while the decreasing in number and length of the kineties as well as a dislocation of the mouth towards the ventral side of the cell suggest a more derived cell morphology.

The layer external to the plasmalemma, called by Faure-Fremiet and Ganier (1970) perilemma, is characteristic for all cells from the suborder Tintinnina (order Choreotrichida) (Laval-Peuto 1975); however, it can also be found in some species belonging to the order Oligotrichida (Bardele 1981). In addition, this layer is present in some genera of the order Stichotrichida (Bardele 1981). It is interesting to note that in tintinnids this layer covers the entire cell body, along with the oral and somatic cilia, while in others ciliates it may be broken at the level of cilia (Laval-Peuto 1975). According to Montagnes and Lynn (1991), the presence or absence of this layer may give important information for phylogenetic relationships. They suggested that choreotrichs and oligotrichs form a monophyletic group with a possible stichotrich ancestor.

Other structures characteristic for the Tintinnina are capsules. Their morphology and size may be used as an additional feature for species identification (Laval 1971). The individual variability in their number depends on the stage in the cell life cycle (Laval 1971). Capsules were described previously as bacteria (Entz 1909), then as trichocysts (Campbell 1926) or trichocyst-like structures (Hedin 1975). Laval (1971) showed their similarity to microtoxicysts. It was also suggested that capsules could play a sensory role or might function as statocysts to maintain balance during fast swimming (Laval 1971). Laval-Peuto et al. (1979) distinguished 3 types of capsules according to their shape and ultrastructure. The capsules in *C. convallaria* seems to belong to the type characterized as being "grossly spherical, with straight conical anterior pool". Laval-Peuto et al. (1979) described the polarization of capsules in relation to the plasmalemma. Our observations confirm capsule polarization for *C. convallaria*, the more so that we observed the presence of fibrillar

structures connecting not only the cap to the capsules but also the cap to the plasmalemma. The function of capsules remains unknown.

The presence of the striae on the adoral membranelles appears to be characteristic of many, but not all, tintinnids (Laval-Peuto et al. 1979). The number of striae per membranelle varies in different species from 2 in *Tintinnopsis parva* (Laval-Peuto et al. 1979), 3 in *C. convallaria* to 10 or more in genera *Petalotricha* and *Cyttarocylis* (Laval 1971, 1972).

The term "morula" is used for a group of osmophilic globules surrounding by the unit membrane (Laval 1972, Laval-Peuto 1975). Such structures are present only in the suborder Tintinnina, but not in all species. In *C. convallaria* the globules forming "morulas" can be loosely to closely packed. The simultaneous presence of differently compacted "morulas" has not previously been reported. Their localization is not constant and changes with the stages of the cell life cycle. In the interphase they are located in the posterior portion of the cell. During division they move towards the anterior part, and thus, after division they are present in the proter. It is suggested that "morulas" are involved in lorica formation (Biernacka 1952, 1965, Hedin 1975, Laval 1972). In tintinnids without "morulas", like *Tintinnopsis parva* or *Parafavella gigantea* (Hedin 1975), this function is fulfilled by lorica-building granules (Laval-Peuto et al. 1979).

In conclusion, although the ultrastructure of *C. convallaria* is typical for the suborder Tintinnina in general, some features seem to be characteristic for this species.

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ACTA
PROTOZOOLOGICA

Effect of Bacitracin on *Tetrahymena*

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Summary. The lysosomal protein (insulin) degradation blocker antibiotic, bacitracin inhibited the insulin imprinting of *Tetrahymena* and direct insulin binding alike. It diminished the glucose activated growth capacity. At the same time the exocytosis of lysosomal enzymes (acid phosphatase, glucosidase and glucosaminidase) increased. The sugar pattern of the plasma membrane changed under the effect of bacitracin treatment, demonstrated by lectin binding. The experiments call the attention to the role of insulin degradation in the insulin imprinting of *Tetrahymena*.

Key words. *Tetrahymena pyriformis*, hormonal imprinting, insulin, bacitracin.

INTRODUCTION

It is essential for the free living unicellulars to feel changes in the environment and to react on them the proper way. To investigate the development of the hormone receptors we use this phenomenon in our experiments, too. Under experimental conditions, molecules suitable for rising signals e.g. hormones, which are present in the surroundings of the ciliate *Tetrahymena* are able to produce changes effecting the further progeny generations. At repeated treatment the changes are detectable in the hormone binding capacity of the membrane (Kovács and Csaba 1990a) and other physiological responses like the phagocytosis (Kovács and Csaba 1990b), growth rate, amount of PAS+ substances (Kovács and Csaba 1989), and the level of the second messenger molecules (Kovács et al. 1989). This

phenomenon is the hormonal imprinting. The phenomenon of hormonal imprinting is well established at phylogenetic levels (Csaba 1980, 1985, 1986), but the mechanism of its development is not clarified yet.

From research of the pathways of insulin action the possibility arose that for some effects it is not the intact insulin but the product of degradation, developed after the internalization (Steiner 1977). On this basis it is supposed that during the development of the hormonal imprinting itself the fragments of insulin molecule can play important roles, too. An other possibility is, that after the ligand degradation (?) the recycling of receptors is needed for the development of reactions characteristic of the hormonal imprinting. To prove these suppositions the following experiments were made. According to the literature the polypeptide antibiotic molecule bacitracin can inhibit degradation of insulin while there is no decrease in the quantity of the cell bound insulin; in certain cases it causes an increase (DeVries et al. 1990). In accordance with the results above we inhibited the degradation of insulin with bacitracin (Plas and Desbu-

quois 1982). In control and bacitracin treated cells the insulin imprinting was studied. We tested the bacitracin effects on the phagocytosis and growth as it was supposed that this agent inhibits not only degradation of insulin but also the lysosomal degradation of proteins and thus influences the normal nutritive processes of cells (the inhibition of the lysosomal function could influence the nutritive processes, too). It is not only the increased pH value - used to explain the effect of lysosomotropic agents - that might be responsible for the inhibition of the activity of endosomal compartments and lysosomes, but also the modification of the synthesis, signalization and sorting of essential enzymes of these compartments. On the basis of the facts mentioned above we investigated whether the bacitracin inhibited the intracellular activity of characteristic lysosomal enzymes of *Tetrahymena* and if it provoked a more intensive secretion of these enzymes into the culture medium. This type of alteration might refer to the altered signalization of these newly synthesized peptides resulted by the changed saccharide signal components. We tested if there is any effect of bacitracin on the lectin binding capacity of the *Tetrahymena* membrane, if it can change the distribution of the surface sugar components. It was done because in prokaryote the bacitracin has an effect on the glycosilation mediated by lipids, and presumably is able to influence all processes where polyisoprenoid pyrophosphate molecules are involved (Parodi et al. 1982, Siewert and Strominger 1967) and can result a modified signalization in eukaryotes.

MATERIAL AND METHODS

The *Tetrahymena pyriformis* GL strain was used in the experiments in the logarithmic phase of growth. The cells were grown at 28°C on 1% Tryptone medium (Difco, Michigan, USA) containing 0.1% yeast extract.

[A] Investigation of the hormonal imprinting

10 ml of culture at density of 3×10^5 cell/ml were treated for 1 hour: (a) treatment with 10^{-6} M insulin (Semilente MC, Novo, Copenhagen, Denmark), (b) treatment with 2mM bacitracin (Sigma Chemical Co., St Louis, USA). Beside these groups combined treatments were also applied, in which insulin treatment was running in the presence of bacitracin. We had absolute control groups (without any treatment), too. After 1 hour treatment the cells were centrifuged and washed by normal medium. 0.5 ml of cell suspension (3×10^5 cell/ml) was inoculated into 10 ml normal medium.

After 1 day the cells were fixed in 4% neutral formalin dissolved in PBS for 5 minutes. The samples were washed trice with PBS. For one hour the samples were incubated at room temperature with FITC

(fluorescein isothiocyanate, isomer I.; BDH Chemicals Ltd., Poole, England) labelled insulin (FITC/protein ratio was 0.37; concentration of protein was 0.05 mg/ml). After incubation the cells were washed with PBS three times and dropped onto microscopic slides. The intensity of fluorescence of the cells was measured by means of a Zeiss Fluoval cytofluorimeter. The analogue signals were converted into digital ones by means of an analogue digital processor. A Hewlett Packard HP 41CX microcomputer registered the signals. Its mathematical program provided the means of the individual groups, the standard deviations and the significance values (Student t-test) between the individual groups.

[B] Measurement of the phagocytotic activity

Populations of *Tetrahymena* cells were separated from the medium by centrifugation and then washed in Losina-Losinsky (L-L) solution. The cells were inoculated into L-L solution for 4 hours. Then the *Tetrahymena* cells were treated with 2 mM bacitracin for 10 minutes. After this incubation Indian ink suspension was added (*Tetrahymena* suspension: bacitracin solution: Indian ink = v : v : v) for 10 minutes. Cells were fixed with formalin and washed in L-L solution. Then cells were dropped onto microscopic slides. The number of the food particles were counted in 100 cells of each group.

[C] Investigations on the rate of multiplication

0.5 ml of cell cultures (4×10^5 cell/ml) were added to 9.5 ml of culture medium. This way the density of samples was 0.2×10^5 cell/ml. In addition to the control groups there were others treated by: (a) glucose (25mM), (b) bacitracin (2 mM) and (c) glucose + bacitracin. The sampling was at the time points shown on the figures. The cell number of the samples was determined in Fuchs-Rosenthal chamber.

[D] Measurement of the lysosomal enzyme activity

Activity of acid-phosphatase, glucosidase and glucose-aminidase enzymes was determined according to Suzuki's method with substrates containing methyl-umbelliferyl (Suzuki 1978). Prior to the determinations the cells were harvested from the culture medium by centrifugation and washed with normal medium. Then the cells were
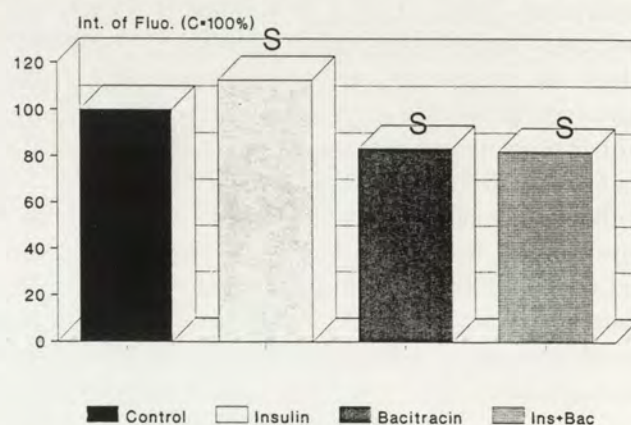
Fig. 1. Binding of FITC-insulin to *Tetrahymena* one day after insulin, bacitracin or combined treatments S = p < 0.01
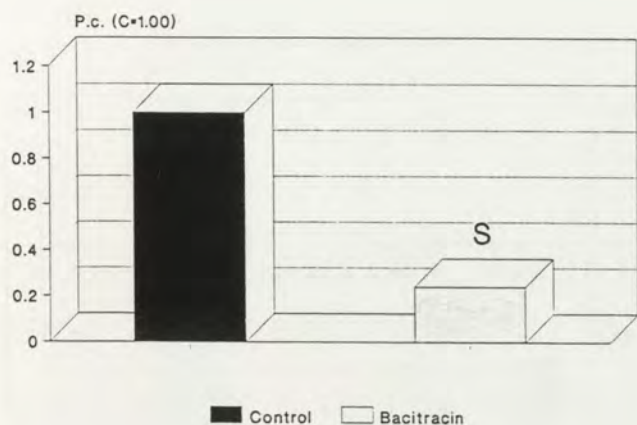
Fig. 2. Phagocytosis coefficient (P.c.) after 10 min bacitracin treatment, related to the control as 100%. S = p < 0.01

placed into normal medium. This cell suspension (density of cells: 5×10^5 cell/ml) was divided into two parts/groups: a) control, b) 2mM bacitracin treated. The cells were then incubated for 1 hour at 28°C. The cells and culture medium were separated by centrifugation and the enzyme activity was determined in both the cells and the medium. The following substrates were applied: (a) 4 methyl-umbelliferyl-phosphate (to acid phosphatase); 4-methyl-umbelliferyl-2-acetamido-2-deoxy-β-D - glucopyranoside (for N - acetyl-D - glucoseaminidase); 4-methyl-umbelliferyl- β-D-glucopyraranoside (for β-D-glucosidase) (Koch-Light Laboratories Ltd., Colnbrook, Bucks, England) Substrates were applied in 5 mM concentration, pH 4.2 in citrate buffer. The intensity of fluorescence of methyl-umbelliferon liberated by the enzyme reactions was determined by Clinifluor 85-M fluorimeter (Izinta, Budapest) with 365nm filter for the excitation and 448nm filter for emission range.

[E] Assay of lectin binding of *Tetrahymena*

The following lectins were applied to assay the sugar components of the surface membrane: (a) Concanavalin A (Con A) (Serva Feinbiochemica, Heidelberg, Germany) Sugar specificity: α-D-Man; α-D-Glc (FITC: protein ratio 1.4), (b) *Helix pomatia* (purified according to Hammarstrom and Kabat (1969) in our laboratory) Sugar specificity: D-GalNac (FITC: protein ratio 1.55), (c) *Phaseolus vulgaris* (purified according to Itoh et al.(1980) in our laboratory). Sugar specificity: oligosaccharides - mainly of NacGal content. (FITC: protein ratio 1.69), (d) *Datura stramonium* (purified according to Kilpatrick and Yeoman (1978) in our laboratory). Sugar specificity: D-GlcNac dimers (FITC: protein ratio 2.07), (e) *Pisum sativum* (purified according to Entlicher et al. 1970, in our laboratory). Sugar specificity: D-Man. (FITC: protein ratio 1.77). The *Tetrahymena* cultures were divided into two parts in the logarithmic phase of growth: one part served as the control group the other was treated with 2mM bacitracin for two hours. Following the treatment the cells were fixed then incubated with the FITC labelled lectins at room temperature for 1 hour. The protein concentration of each FITC labelled lectins was 0.1 mg/ml. After incubation the cells were washed in PBS three times, then dropped onto microscopic slides.
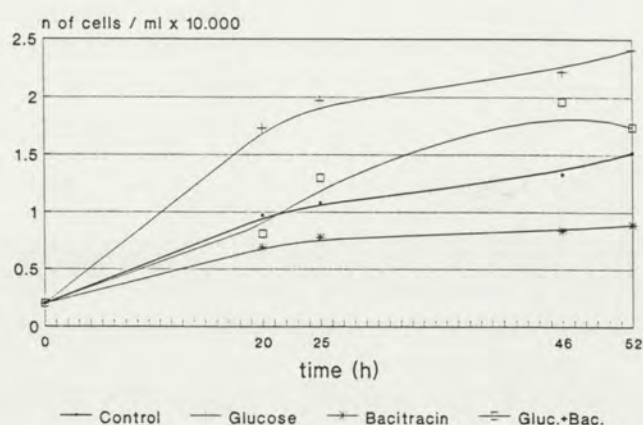
Fig. 3. Growth activity of control, glucose or bacitracin treated and jointly treated *Tetrahymena* plotted against the time

The intensity of fluorescence was determined. Along with the lectins the FITC labelled insulin binding was also determined.

RESULTS

On the following day of insulin imprinting the binding of FITC-insulin increased significantly above the level of the untreated, control group (Fig. 1). After one day of the treatment with bacitracin - about 6 new generations were developed - we could register a significant decrease of the insulin binding. In the presence of bacitracin the insulin did not result an increased binding which is characteristic of imprinting. We measured a significantly decreased capacity of insulin binding immediately after the bacitracin treatment for two hours (Fig. 5). The activity of phagocytosis was reduced very significantly by bacitracin (Fig. 2). The growth activity of the *Tetrahymena* cultures was stimulated by the 25 mM glucose. Bacitracin (from the beginning) significantly diminished the growth activity but the presence of glucose in the culture medium could compensate this effect, mainly after the 20th hour of the experiment (Fig. 3). By the applied methylumbelliferyl substrates we could detect an increased acid phosphatase activity following the bacitracin treatment (p < 0.01) (Fig. 4a). On the other side there was no considerable change in the cells after treatment. The bacitracin results in a significant increase of glucosidase and glucosaminidase levels in the culture medium (p < 0.01) (Fig. 4b and c). The treatment with bacitracin decreased binding of insulin significantly compared to the control cells (Fig. 5). The Con A and PHA binding is increased significantly (p < 0.01); while this increase is moderate

but significant in the case of *Datura* lectin (p < 0.01) after the bacitracin treatment. There was a significant drop in the binding of *Helix* lectin (p < 0.01).

DISCUSSION

Bacitracin is an inhibitor of protein degradation. This way it inhibits the degradation of insulin, too, but there is no inhibition on the binding sites of insulin or internalization of the hormone (DeVries et al. 1990, Plas and Desbuquois 1982, Yagil et al. 1988). Presumably the bacitracin has an effect also on the lipid-dependent glycosylation (Siewert and Strominger 1967) not only in prokaryote but in unicellular eukaryotes (where the dolichol-phosphate - like in the higher eukaryotes - serves as an oligosaccharide donor). It also influences inhibition the glycosilation of lysosomal enzymes (though the mechanism of glycosilation differs from the higher eukaryotes, somehow (Parodi et al. 1982). The acid phosphatase enzyme contains 10% (referred to weight) carbohydrate (Banno and Nozawa 1984).

In mammalian cells the most lysosomal enzymes are glycoproteins (Hasilik and Neufeld 1980). The inhibitory effect of bacitracin could stop the initial steps of the insulin degradation and drive the intact insulin molecules or the early products of degradation to the "retroendocytotic pathway" in cells of kidney epithelial cell-line (Dahl et al. 1990). All these results suggest that the degradation of insulin started on a prelysosomal way. Presumably it runs already in those endosomes where the pH is not decreased and the insulin is not dissociated from the insulin receptor (Hamel et al. 1991). In a cell-free system the bacitracin inhibits also the enzymatic activity of isolated endolysosomes (Doherty et al. 1990). So we have to suppose that not only the inhibition of the lysosomal enzyme synthesis but the inhibition of enzyme activity is responsible for the effects of bacitracin. Though there are also data that bacitracin does not inhibit the lysosomal enzyme activity (Plas and Desbuquois 1982). Our results derived from *Tetrahymena* experiments do not agree with the results mentioned above in many respects. The 2 hour bacitracin treatment decreased significantly the binding of insulin (Fig. 5). This fall in binding was significant following 1 day of the one hour treatment. Insulin could not develop imprinting in the presence of bacitracin (Fig. 1). The changes in the composition of the plasma membrane are characterized by the great increase of the lectin binding capacity of the plasma membrane (Con A; PHA
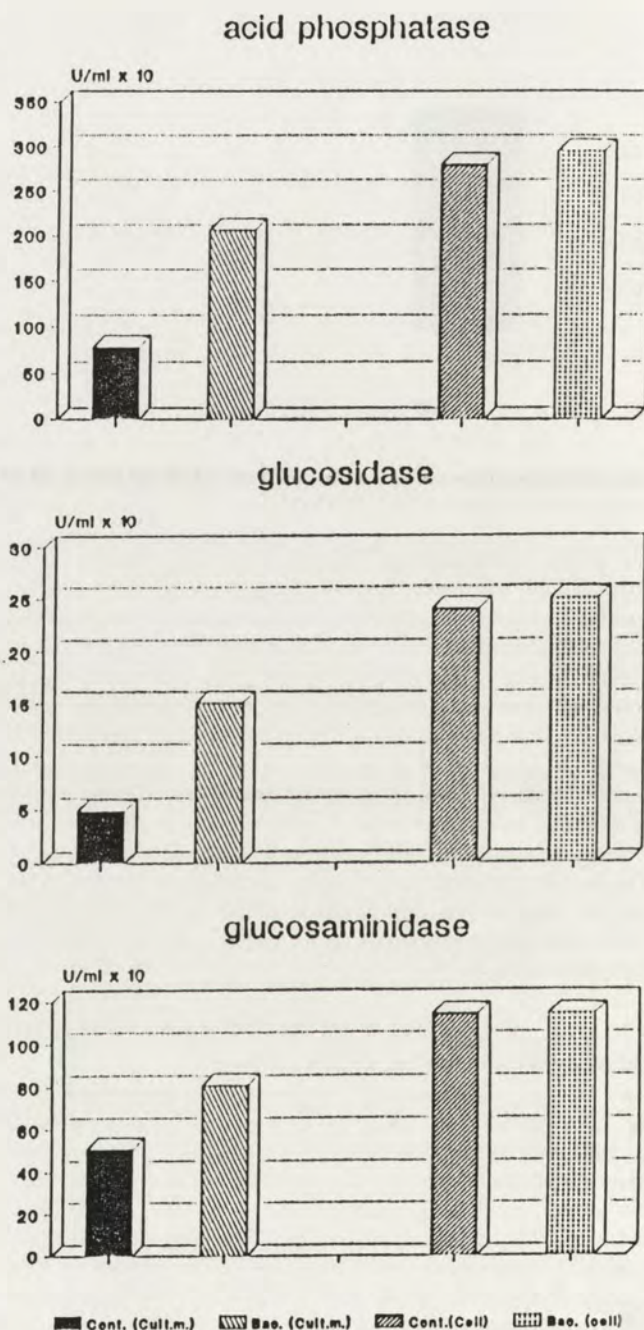
Fig. 4. Lysosomal enzymes in control and bacitracin treated cells and in the culture media

and *Datura* in a moderate manner), only the GalNac specific binding of the *Helix* lectin decreased below the control level (Fig. 5). Presumably this altered composition of the membrane is also responsible for low phagocytotic activity measured in the presence of bacitracin (Fig. 2). The reduced phagocytotic activity after bacitracin treatment could be evoked theoretically by different factors: the bacitracin can influence the

function of lysosomes directly (thought there are more disproving data of literature e.g. (Plas and Desbuquois 1982); can inhibit the synthesis of the lysosomal enzymes and the transport to the lysosomal compartments; by the altered glycoproteins of the surface there is an inhibition of the first step of endocytosis the recognition of the food molecules by the membrane. Presumably bacitracin is able to develop such kind of effects which are characteristic for the starvation by its capacity to inhibit formation of food vacuoles. Our previous experiments demonstrated that insulin evoked imprinting did not develop in *Tetrahymena* cells during starvation and the insulin binding capacity was also decreased in these conditions (Csaba et al. 1992). The measured alterations were similar to the bacitracin induced ones. In the synthesis of the lysosomal enzymes the carbohydrate molecule components have an important role as signals in the transport of these molecules to the lysosomes (Hasilik and Neufeld 1980). If bacitracin interferes with the glycosilation of these molecules (Siewert and Strominger 1967) we can explain the increased secretion of lysosomal enzymes to the culture medium in the presence of bacitracin. The changed binding of lectins refers also to the qualitative alteration of glycoproteins built into the plasma membrane. The disturbance of the signalization might lead to a changed sorting of glycoproteins in the Golgi complex resulting that certain glycoproteins will be placed to an inadequate site. Moreover the Golgi apparatus can influence the membrane recycling in bacitracin treated cells. The inhibition of glycosilation can inhibit the normal process. It is possible that following the insulin binding on the surface membrane and internalization the structures possessing a special binding capacity can not recycle. On one hand this can inhibit the binding of insulin further on, on the other hand - because of the reduced uptake of food - this will lead to a decreased multiplication, too. Bacitracin reduced the multiplication rates of *Tetrahymena* (Fig. 3b), but in the presence of glucose there was an increased multiplication in the bacitracin treated group. Although this can be explained by one more or less doubling of the participating cells, it was consistent in the whole period of measurements. According to this it is presumable that bacitracin does not inhibit the glucose utilization. On the basis of our results we can conclude - as we have already supposed according to our previous experiments - that the disturbance of the system which is responsible for binding of hormone and transmission of effect towards the effector systems do not make the development of hormonal
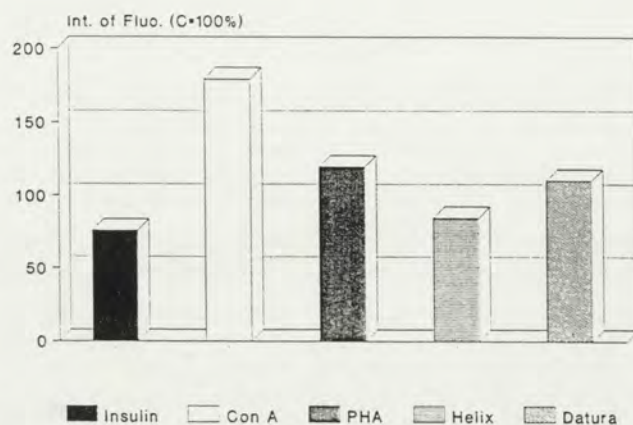
Fig. 5. Binding of FITC-insulin and FITC-lectins to *Tetrahymena* treated with bacitracin for two hours, related to the control as 100%. (Con A - concanavalin A; PHA - phytohaemagglutinin)

imprinting possible. This way the alteration of surface proteins, the surface receptor-pool (fall in insulin binding capacity) did not let hormonal imprinting develop. Our present and other experiments lead to the conclusion that the inhibition of insulin degradation has also a role in the absence of hormonal imprinting. Lidocaine can decrease the insulin degradation in hepatocyte (Terris et al. 1979) and in the presence of it the insulin imprinting failed (Nozawa et al. 1985). Also cycloheximide can inhibit the degradation of lysosomal proteins (Hopgood et al. 1981) and it did not make possible to develop hormonal imprinting neither in *Tetrahymena* (Köhidai et al. 1985). This is the situation in the case of chloroquine, too (Kovács and Csaba 1988). Obviously the mentioned materials affects not only degradation but if we study these results together there is no reason to preclude the possibility that the inhibition of degradation is responsible for the failed imprinting, too. In the literature there is a theory that points to that: there are certain characteristic effects of insulin which are produced mainly by degradation components associated with the membrane (Plas and Desbuquois 1982).

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