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Review article

Endocytosis, Digestion, and Defecation in Flagellates*

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INTRODUCTION AND TERMINOLOGY

Nutritional strategies of protists are extremely diverse. This review concerning flagellates focuses on the mechanisms of acquisition and uptake of particular food (phagocytosis), on the principles of digestion, and finally on the defecation of digestive residues.

In principle, autotrophic, green protists (flagellates) containing chloroplasts are regarded as plants and heterotrophic, colorless species as animals. Within one systematic group there may be forms of both types of gaining nutrients indicating a close evolutionary relationship of animal- and plant-like protists. The required organic components may be obtained by uptake of living or detrital particles (phagocytosis), by uptake of dissolved nutrients either by pinching off small sized vesicles (pinocytosis) or directly through the plasma membrane (osmotrophy), by metabolic exchange with endosymbionts or through combinations of these mechanisms (Nisbet 1984). The way the food is detected, collected, concentrated, and internalized varies between the different taxonomic groups or even species and depends on the food properties. Different nutritional types can be classified according to the organisms' sources of carbon, energy, and reducing equivalents. Autotrophs use only inorganic compounds (CO_2) for photosynthesis, heterotrophs require organic compounds for metabolism. Heterotrophic organisms containing functional chloroplasts are mixotroph. Energy may be acquired by (sun) light (phototrophy) or by oxidation of organic or inorganic compounds (chemotrophy). Lithotrophs use inorganic, organotrophs organic sources as reducing equivalents for cell metabolism. Organic substances may be taken up by phagocytosis of solid food particles (phagotrophic or holozoic nutrition) or by intake of dissolved substances (osmotrophic or saprozoic nutrition). Phagotrophic protists sort among potential prey using physical parameters such as particle size and shape (Monger and Landry 1991). Additional chemosensory capabilities have been reported for numerous ciliates and flagellates (Verity 1991).

Ingestion of food particles may be realized via invagination or pseudopod formation of a slightly differentiated region of the plasma membrane or by specialized feeding apparatuses. The terms used to describe the oral

*Dedicated to **Prof. Dr. P. Sitte**, University of Freiburg, Germany, on the occasion of his 65th birthday.

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apparatuses have no uniform meaning in the literature. We define them in the following sense (Fig. 1). An invagination into which food particles are drawn and which is supported by cytoskeletal elements is generally called buccal or oral invagination. If the invagination is voluminous it is a buccal or oral cavity whereas channel-like feeding apparatuses are called feeding-tubes. At its closed end the cytostome whose membrane is deprived of cortical structures pinches off food vacuoles. Microtubular or filamentous fibrils may conduct the vacuoles deeper into the cytoplasm. They form a cytopharynx. Frequently, the fibrils are continuous with the cytoskeletal elements bordering the buccal invagination.

Protozoa need not strictly follow one type of nutrition. Mixed forms exist and responding to environmental conditions nutrition strategies can vary according to the requirements. In darkness, e.g., an autotrophic organisms (e.g., *Euglena gracilis*) may lose its chlorophyll and switch to heterotrophic nutrition (Pringsheim 1963). Heterotrophy is found in several so-called algal groups, the Cryptomonadida, Dinoflagellida, Euglenida, Chloromonadida, Chrysomonadida, and Prymnesiida. Phagotrophy, in particular, seems to be an unexpected but widespread phenomenon within green forms (Bird and Kalff 1987). Phagotrophy was, e.g., reported for genera as *Chrysochromulina, Chlorochromonas, Phaeaster, Pedinella* and *Ceratium*. Volvocida, Prasinomonadida, and Silicoflagellida do not feed on particulate material. Uptake of particulate food not only by colorless but also by green protists adds a further complication to studies of food web dynamics in freshwater and - in all likelihood - in marine habitats, as well (Bird and Kalff 1987). This phenomenon can, however, account for the occurrence of deep water layers with a low light intensity possessing a high chlorophyll content, i.e., a dense population of algae. They probably survive by phagotrophy rather than by photosynthesis alone. Apart from the advantage to receive organic carbon for energy in the absence of light, phagotrophic green cells can obtain minerals in a concentrated form, especially phosphorus in freshwater and nitrogen in the sea (Boraas et al. 1988). The ecology of marine protozoa and in particular their feeding-related control has been recently reviewed by Capriulo (1990). Sanders and Porter published in 1988 a general review about the ecology of phagotrophic phytoflagellates.

Pure autotrophy will not be discussed in this review but instead we will focus on a variety of heterotrophic mechanisms found in flagellated protozoa. Since there is no single regime of feeding that is realized by all systematic groups we proceeded in describing the different mechanisms separately for each order of flagellates following the systematics of Levine et al. (1980). Readers especially interested in systematics of protists are referred to the recently published up-to-date classification by Corliss (1994).

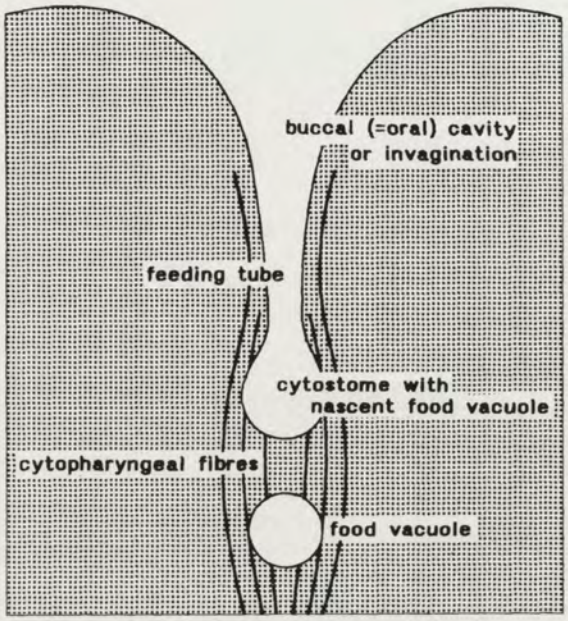

Fig. 1. Scheme of a buccal (= oral, = feeding) apparatus with definition of terms. (After Hausmann and Radek 1993)

Cryptomonadida

There are known pigmented and colorless species of the single suborder Cryptomonadina. Two mastigoneme-bearing flagella arise from an apical or ventral groove or tube-like invagination called gullet. Nutrition has been little studied. A function of the gullet in food uptake is controversially discussed. According to Kugrens et al. (1986) phagocytosis, if it occurs at all, is brought about by the edges of a ventral furrow closing over the prey thus forming the gullet. Mignot et al. (1968) speculate that at least some part of the groove is capable of endocytosis. Santore and Leedale (1985), however, state that the gullet does not seem to be active in phagocytosis at all. In spite of several reports of food vacuoles containing bacteria in cryptomonads direct documentation of the ingestion process has not been delivered (Boraas et al. 1988, Mignot 1965, Porter et al. 1985, Schuster 1968).

Ultrastructural evidence for bacterial ingestion was given for the green cryptomonad *Chroomonas pochmanni* (Kugrens and Lee 1990). The organisms selectively capture a particular type of bacteria and retain it in a large vacuole in the anterior part of the cell. The vacuole could be considered an oral cavity. The bacteria are drawn into the vacuole through a small aperture, which can be closed by fusion of the vacuolar membrane. Supporting fibrils, however, are missing. The nearby Golgi apparatus does not add material to the vacuole and digestion does not seem to take place here. A symbiotic function of the enclosed bacteria is discussed. It may be only the non-healthy bacteria, recognizable in the electron microscope by a darker staining, that are removed and digested in smaller vacuoles. The staining intensity and degradation of the ingested bacteria increase towards the posterior part of the cell. The vesicles and their degenerating contents become concentrated closer to the nucleus.

Another species, *Goniomonas truncata*, ingests bacteria through a special opening and obviously digests them in food vacuoles (Kugrens and Lee 1991). Also some species of *Cryptomonas* have been suggested to be capable of phagocytosis (Tranvik et al. 1989, Wawrik 1970). Other heterotrophic phagocytozing flagellates such as *Katablepharis/Kathablepharis* and *Leucocryptos* that have been placed in the cryptomonads are not included in that systematic group any longer (Vørs 1992).

Dinoflagellida

Despite the fact that dinoflagellates are in all likelihood a monophyletic group, they differ greatly in morphology and ecology. They are widely distributed in marine and brackish water and some are even symbionts or parasites. About half of the known species are heterotrophs. For further information concerning the biology of dinoflagellates see Taylor (1987). The nutritional strategies of dinoflagellates were recently reviewed by Elbrächter (1991a) and Schnepf and Elbrächter (1992). The significance of fecal pellet production was discussed by Elbrächter (1991b). Since the authors already comprehensively treated phototrophic, mixotrophic and heterotrophic mechanisms we will only present the main types of phagocytotic food uptake in short.

Ingestion of discrete particles is reported for armored (thecate) dinoflagellates which possess cellulose thecal plates within their pellicular alveoli (= amphiesmata) as well as for athecate species. Some species apparently are specialized on certain food; mostly small, green flagellates are preferred. Prey recognition may involve sensitivity to dissolved organic compounds (Spero 1979). In most cases not the process of ingestion itself but only the presence of food vacuoles as an indication for phagocytosis has been observed. Ingestion typically occurs at the junction of the flagellar grooves (*Glenodinium pulvisculus, Gymnodinium marinum*) or at the posterior of the cell (*Gymnodinium rubrum, Protoperidinium globulus*) (Gaines and Elbrächter 1987). Thecate species (*Peridinium gargantua, Gymnodinium helveticum*) may take up food particles enclosed by a pseudopod by dilatating the cell body or sulcus (Popovský 1982, Pringsheim 1963). Many armored dinoflagellates, however, digest enclosed large prey cells outside the cell body proper since the sulcal opening is too narrow to internalize large particles (Jacobsen and Anderson 1986). Gaines and Taylor (1984) documented an unusual kind of extracellular digestion for *Protoperidinium conicum* and *P. depressum*. These organisms extrude a pseudopodial so-called feeding veil through the flagellar aperture that surrounds the prey (Fig. 2). The prey, preferentially diatom cells, is gradually digested within 20 to 30 minutes but is not drawn inside the theca. The veil is retracted after completion of digestion and the dinoflagellate resumes swimming.

Schnepf and Deichgräber (1984) called a special type of nutrition myzocytosis. Here, the prey is pierced by means of an extensible, tube-like peduncle and the contents are 'sucked out', including both dissolved and particulate organic substances. This mechanism is found in many ectoparasitic forms, e.g., *Gymnodinium* and *Paulsenella* (Schnepf et al. 1985). Another kind of appendage is the so-called stomopod. *Erythropsidinium agile* or *Amylodinium*, e.g., possess such a slender, sharp rod that appears to inject lytic substances into its prey (Greuet 1969, Lom and Lawler 1973). In a feeding *Amphidinium cryophilum* a feeding tube called phagopod emanates from the posterior cell pole (Wilcox and Wedemayer 1991). It is a hollow cylinder that does not contain cytoplasm in its lumen. When a phagopod pierces the prey cell theca food material passes through the phagopod into a nascent food vacuole. The phagopod is left behind after feeding. The newly discovered genus *Schizochytriodinium* infests copepod eggs and protrudes a peduncle-like organelle into the egg (Elbrächter 1988). An enlarging hyaline area appears around the organelle,

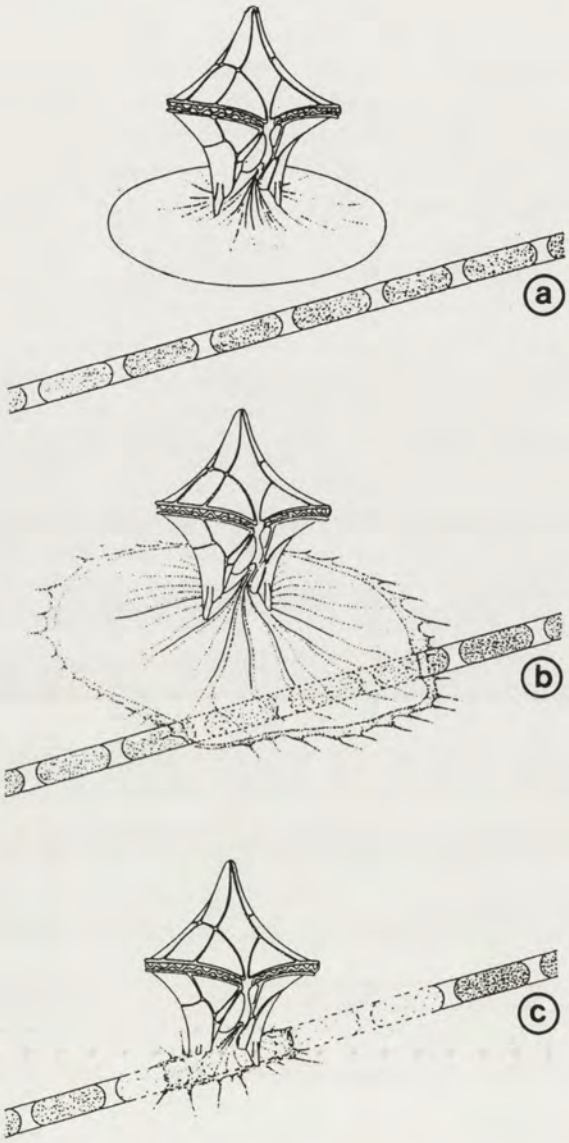

Fig. 2. Different stages (a, b, c) of a *Peridinium* attacking a chain of diatoms. A feeding veil is extruded and surrounds regions of the prey. (After Gaines and Taylor 1984)

implicating extracellular digestion taking place. Another kind of extracellular digestion with subsequent uptake of the dissolved products is called saprotrophy. Here, cytoplasmic threads emerge from the thecal pores of the dinoflagellate. They anastomose and form a delicate network which entangles algae and small nanoplankton to digest them.

Besides different appendages as feeding organelles some dinoflagellates, e.g. *Kofoidinium, Pratjatella*, and *Craspedotella* possess permanent cytostome-cytopharyngeal complexes (Cachon and Cachon 1969). The omnivorous *Noctiluca* uses its tentacle to catch prey and bring it into contact with the cytostome (Oami and Naitoh 1989, Uhlig 1972). The membrane of the cytostome invaginates to enclose the food after cytoplasm has aggregated toward the cytostome (Nawata and Sibaoka 1983). The bioelectric control of food intake behavior of *Noctiluca* was studied in detail by Oami and Naitoh (1989) and Nawata and Sibaoka (1987). For example, external Ca^{2+}, Mg^{2+}, and Cl^- ions are necessary for the induction of the food intake behavior. The external Ca^{2+} presumably is driven into the cytoplasm during membrane hyperpolarization and regulates the cytoplasmic streaming essential for food ingestion.

Euglenida

Most euglenoid flagellates are freshwater organisms but marine or parasitic species also occur. Characteristic features are: two flagella arising within an anterior invagination comprised of a tubular canal and a pyriform reservoir, proteinaceous pellicular strips and eyespots (stigmas). Both colorless and chloroplast-bearing species are common. Colorless species are phago- and/or osmotrophic while green ones are mainly photoautotrophic. Some of the latter are facultatively heterotrophic but do never feed on particulate material (Leedale 1985).

Pinocytotic uptake of molecules from the environment takes place at the single membrane that lines the reservoir cavity (Kivič and Vesk 1974). Colorless forms in general live in a medium with a high content of soluble, organic compounds, e.g., sewage. Leedale (1967) divides the order Euglenida into six suborders of which only the Sphenomonadina and the Heteronematina contain colorless, phagotrophic genera. In contrast to the Sphenomonadina the Heteronematina possess special ingestion organelles.

The feeding apparatuses of the euglenids can be grouped into four types by means of their morphological characteristics (Triemer and Farmer 1991). Type I is a simple cytoplasmic pocket extending posteriorly. It is lined by microtubules all along its length which in part are probably derived from a microtubular flagellar rootlet. Anteriorly the feeding pocket merges with the flagellar pocket. Numerous small vesicles border one side of the feeding apparatus. Type I feeding apparatuses occur in bacterivorous euglenids such as *Petalomonas cantuscygni* and *Calcimonas* sp. and also in *Diplonema*.

In type II the oral apparatus is supported by two rods which extend into the cytoplasm and a series of vanes.

The rods contain few microtubules and eventually a dense amorphous matrix. The vanes surround the invagination of the plasma membrane which forms the cytostome anteriorly and accompany the rods. Vanes and rods are intimately associated at the base of the feeding apparatus. Type II apparatus is restricted to bacterivorous species as *Serpenomonas costata* (= *Ploeotia costata*, Triemer 1986), *P. vitrea* (Farmer and Triemer 1988a), and *Diplonema* (Porter 1973, Schuster et al. 1968b, Triemer and Ott 1990).

Organisms with type III feeding apparatuses are capable of engulfing eukaryotic prey, e.g., *Peranema trichophorum* (Nisbet 1974), *Dinema sulcatum* (Farmer and Triemer 1988b), and *Anisonema* sp. (Triemer 1985). Here, in contrast to type II, the two supporting rods are mainly composed of microtubules and the four vanes are not closely apposed to the rods all along their length. The vanes arise from the rods at the base of the feeding apparatus but diverge anteriorly. Further fibrillar components may be associated with the oral apparatus. The structure and possible mode of operation of the type III feeding apparatus has been extensively examined in *Peranema trichophorum*. Nisbet (1974) proposed a model of its complex, protrusive feeding apparatus and worked out a hypothesis for its movement. The oral invagination and the reservoir have a common opening via a short anterior canal. The oral invagination is supported by a light microscopically visible double rod-organelle of which each rod is composed of a core of interlinked microtubules surrounded by an electron lucent matrix and a sheathing membrane. The rod-organelle is speculated to be pulled forward by contraction of longitudinal lamellae which are attached to the base of the rods and to the opening of the anterior canal. Cytoplasmic pressure may also be involved in force production. The entire feeding organelle moves forward, expands the anterior canal and by withdrawal 'sucks' food into the cytostomal sac. According to Chen (1950) the pellicle near the anterior ends of the rods is adhesive for prey. Ingesta do not pass into the blind-ending reservoir because the passage to the reservoir is closed by the moving rod-organelle. According to Mignot (1966) vesicles with granular contents underlying the cytostomal sac suggest some external digestion and pinocytotic uptake of nutrients in addition to degradation within food vacuoles. Nisbet (1974) denies piercing of the prey by the rod-organelle but postulates operation through adhesion and cytolysis.

A great variety of organisms can be ingested by *Peranema* provided they are motionless. Prey is swallowed or when it is wounded preferentially 'sucked out' (Chen 1950). The food vacuoles of *Peranema* gradually decrease in size without passing through a swollen phase until they are absorbed. Feeding with yeast cells stained with pH indicators revealed an unusually high pH of 5.8 to 6.8 in the food vacuoles. Starch grains, oil droplets and protein particles were shown to be engulfed and apparently digested (Chen 1950). Small remains of digestion may be discharged by *Peranema* through an area of defecation.

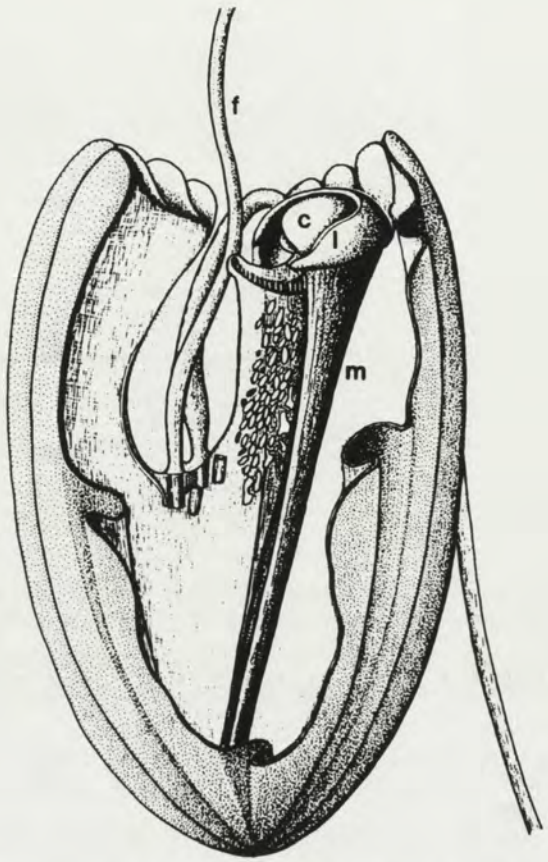

Fig. 3. Tube-like feeding apparatus (siphon) of *Entosiphon sulcatum*. Semicircular lips (l) cover the anterior ends of the microtubular rods (m). A central cap (c) can be withdrawn and thus opens the siphon during protrusion of the feeding organelle. f – flagellum. (After Mignot 1966)

Type IV oral apparatus is only found in the bacterivorous *Entosiphon sulcatum* (Mignot 1966, Triemer and Fritz 1987). It possesses a tube-like feeding apparatus (siphon) extending the length of the cell which can be moved forward and backward for a distance of 3-5 µm (Fig. 3). Within the tube of *E. sulcatum* four striated vanes arise from three microtubular rods (instead of two rods as typical for type III) like the blades of a pinwheel. At the anterior end of the siphon the vanes

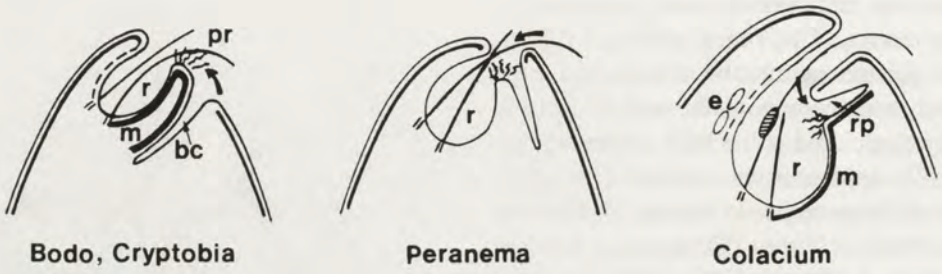

Fig. 4. Schemes of longitudinal sections through the flagellar pockets or reservoirs (r) of Trypanosomatida (*Bodo, Cryptobia*) and Euglenida (*Peranema, Colacium*). The reservoir pocket (rp) of euglenids which seems to be a rudimentary oral apparatus may have developed from the respective trypanosomatid organelles by coalescence of the two openings combined with an invagination. bc – buccal cavity, m – microtubular ribbon, pr – preoral ridge. (After Willey and Wibel 1985a)

converge and surround an elongate invagination of the plasma membrane which extends downward into the tube. The vanes are connected to the plasma membrane at the apex. A cap covers the opening of the siphon. The cap is withdrawn and thus opens the siphon during protrusion of the feeding organelle. During extension the vanes spread apart expanding the invagination to a large cavity into which food particles can be drawn. Endocytotic vesicles are formed and seem to be pushed down the apparatus. Triemer and Fritz (1987) established a hypothesis for the movement of the feeding organelle based on sliding of rod microtubules. According to this hypothesis two groups of anti-parallel orientated microtubules allow movement in analogy to the half-spindles in mitosis. Concomitant to microtubule-sliding the vanes which are attached to the microtubules to most of their length may spread apart like the leaves of an iris diaphragm thus widening the invagination to a cavity. However, according to Willey et al. (1988) the sliding microtubule model appears inadequate since Lackey (1929) reported that the feeding organelle does not change in length during ingestion and is rigid and non-contractile.

Most euglenid species do neither possess an oral invagination nor a complex feeding organelle. However, Surek and Melkonian (1986) discovered a cryptic cytostome in *Euglena mutabilis*. It consists of a short cylindrical infolding which arises from the ventral surface of the reservoir and is linked to flagellar root microtubules. Coated pits suggest occurrence of endocytosis. The authors see a homology between the reservoir pocket of *Euglena* and the cytostome-cytopharyngeal complex of Kinetoplastida, supporting the phylogenetic derivation of Euglenida from Kinetoplastida and of green euglenids from phagotrophic, colorless taxa (Fig. 4). The theory was sustained by the discovery of similar reservoir pockets in *Colacium, Trachelomonas, Euglena acus* and *E. gracilis* (Willey and Wibel 1985a, b). The pocket membrane of *Colacium* is covered with a microfilamentous mesh and is bound to a band of microtubules, which appear to brace the pocket membrane by microfilamentous strands. The pocket is ultrastructurally close to the cytostome-cytopharyngeal complex of the bodonids. Phylogenetic reduction of the bodonid ingestion apparatus to the euglenoid reservoir pocket may be accomplished by rotation and invagination. Willey et al. (1988) discuss the possible origins of the euglenoid phagotrophic apparatus in detail. The presumptive oral pockets of the euglenoids seem to be rudimentary endocytotic or exocytotic organelles rather than being feeding organelles actively engulfing prey. Smooth and coated vesicles alongside the pocket of *Eutreptia pertyi*, e.g., are speculated to be involved in endocytosis and digestion (Solomon et al. 1991). Comparable pockets have been found in euglenids that in addition possess more elaborate feeding apparatuses, e.g., *Serpenomonas, Dinema*, and *Diplonema* (Triemer and Ott 1990). Triemer and Farmer (1991) speculate that these pockets, possibly deriving from some ancestral feeding apparatus, are retained for pinocytotic functions in addition to the phagotrophic feeding apparatus.

Chrysomonadida

The chrysomonads are basically biflagellate cells with one flagellum bearing mastigonemes. Most species may also become amoeboid. The majority is naked though also distinct cell surface structures such as scales or loricae may exist. The chrysomonads bear affinities both to plants and animals. There is a strong tendency toward complete loss of chlorophyll. Most seem to be phagotrophic in nature, e.g., *Chrysophaerella, Uro-*

glena, Catenochrysis, Ochromonas, Chromulina, and *Chrysococcus*. The genera *Synura* and *Mallomonas* appear to be an exception (Bird and Kalff 1987). Food particles are trapped in a variety of ways involving flagellar action aided by pseudopodial ingestion and often a protoplasmic collar helps to concentrate the prey (Nisbet 1984).

The intensively studied genus *Ochromonas* is a good example for nutritional diversity of the group. It is capable of phagotrophy, heterotrophy, photoheterotrophy and photoautotrophy (Aaronson 1974). *O. danica* is quite unselective in its ability to use various food organisms. It may feed on several bacterial species, yeast, or cyanobacteria. Sometimes, cannibalism occurs. Capture of food is aided by the flagellar action producing water flows which bring particles and soluble food into contact with the surface of the organism (Aaronson 1973). A maximum flow velocity adjacent to the flagellum of 0.25-0.3 mm/s is reached when *Ochromonas* cells feed on particles 4-5 µm in diameter (Boraas et al. 1988). Particles are trapped at the surface and may accumulate into a cloud around the cell. Only living cells are capable of aggregating particles indicating a necessity of metabolic activity to accomplish this. Secretion of a variety of molecules and of vesicles which pinch off from surface projections seem to be responsible for sampling of the environment by aggregation. Aaronson (1973) presumes that molecules and vesicles may act as adhesive or trapping material or may serve to attract food organisms, so that food may be phagocytozed or pinocytozed.

Food particles are taken into food vacuoles (primary endosomes) at the anterior end or at the sides of the organisms (Aaronson 1973). Schuster et al. (1968a) designates the anterior region of feeding a cytoplasmic tongue differentiated by fibers arising from the kinetosomes and by pinocytotic activity. This specialized region does not necessarily imply selectivity of feeding. *O. malhamensis* engulfs bacteria as well as undigestible, biochemically inert latex particles and even adds hydrolytic enzymes to the food vacuoles (Dubowsky 1974). The author supposes that the only requirement to stimulate ingestion is the appropriate size and weight. *Ochromonas* does not, however, ingest particles which are smaller than 3 µm (Boraas et al. 1988). Bacteria and beads of that size may be captured but are then released. *Dinobryon*, as well, was shown to be incapable of ingesting very small particles (0.28 µm, Bird and Kalff 1987). Feeding of *Ochromonas* species may not only be specific for size but also for shape (Boraas et al. 1988). Spherical shapes seem to be preferred to irregular, spindle- or lunate-shaped forms. Food particles of the latter type cannot sustain growth of an *Ochromonas* population.

Two bundles of microtubules lining the right margin of a ventral furrow of *Ochromonas* seem to mediate the phagocytosis of bacteria. When a bacterium touches that region the right margin rises to a 2 µm high concave wall in which the food particle is trapped by enclosure (Fenchel 1982). A feeding *O. danica* retracts its tail and assumes a spherical shape. Cole and Wynne (1974) suggest that endocytosis has a disruptive effect on the anterior microtubule nucleating sites of the microtubular system underlying the cell surface. Disassembly of the skeletal system, supported by a high turgor pressure established by the enlarged secondary endosome, may evoke tail retraction and spherical shape.

Food vacuoles formed by *O. danica* migrate to the posterior region where they coalesce to form a large digestive vacuole (secondary endosome). According to Aaronson (1974) this vacuole grows enormously, discharging and compressing an adjacent leucosin vacuole. In contrast, other authors think that the same posterior vacuole may store leucosin and assume an alternate function as a secondary endosome (Cole and Wynne 1974, Daley et al. 1973). In any case, new, small food vacuoles empty into a large posterior vacuole. Bacteria in various stages of digestion are found therein (Aaronson 1974). Digestion is accompanied by loss of bacterial electron density and an increase in vesicle and membrane formation in the food vacuole. According to Cole and Wynne (1974) digestion of the cyanobacterium *Microcystis aeruginosa*, which is toxic for other animals, starts simultaneously when the filling of the posterior vacuole reaches its full capacity. At this stage no further engulfment of prey occurs. Thus, an initial rapid uptake of cells is followed by a digestive period. According to Daley et al. (1973) digestion is not fully accomplished in the posterior vacuole but carried on in smaller digestive vacuoles which pinch off. Probably, at least digestion of the outer mucilaginous sheath of *M. aeruginosa* already begins within the primary endosomes. Vesicle populations which contain membrane fragments and diffuse material surround both the primary and secondary endosomes. Additionally, coated vesicles appear in the region of the posterior vacuole. They are thought to arise either by specialized micropinocytosis, since coated pits can be demonstrated in the plasmalemma or from Golgi cisternae. Kahan et al. (1978) confirmed the production of coated vesicles by the Golgi apparatus

which seem to pass to and fuse with the cell membrane. Lamellae of endoplasmic reticulum frequently surround the secondary endosome. The endosomal membrane is highly convoluted. Blebbing and fusion seem to occur at its surface. Reaction products of acid phosphatase were found in Golgi vesicles, larger vesicles surrounding the secondary endosome and the secondary endosome itself (Cole and Wynne 1974). Digestion seems to follow the typical scheme, in short: fusion of primary endosomes with enzyme-delivering vesicles (primary lysosomes) arising from Golgi apparatus or ER, resulting in a secondary endosome and subsequent vesicles pinching off. Various hydrolytic enzymes have been found in *O. danica* and several may be secreted (Aaronson 1973). Certain types of surface blebs and vesicles may serve as a mechanism for secretion (Kahan et al. 1978). The effect of the secreted protein on the food organisms is unknown. Membranous material and other residues accumulate in the vacuoles during digestion. These vacuoles converge to residual bodies which may be transformed into lipoidal deposits or are discharged by exocytosis (Cole and Wynne 1974). *O. malhamensis* does not seem to reclaim its digestive enzymes prior to exocytosis. They are expelled along with the defecated material (Dubowsky 1974).

Cole and Wynne (1974) suggest exocytotic activity in the chrysomonad *Ochromonas danica* by demonstration of evaginations on the surface of freeze-etched cells. Dense remnants of digested cyanobacteria and experimentally fed latex particles were shown to be egested (Daley et al. 1973, Dubowsky 1974).

Little is known about phagotrophy in photosynthetic chrysomonads. *Uroglena americana* is another green phagotrophic chrysomonad (Kimura and Ishida 1985). *Uroglena* is unable to grow in the light without ingesting bacteria. Bacterial phospholipids seem to be essential for growth.

Many chrysomonads form cup-like feeding apparatuses or food baskets near the basal bodies during ingestion (Moestrup and Andersen 1991). When a prey cell has been arrested by the flagella the feeding cup extends and engulfs the food. In *Epipyxis pulchra* this process takes 2-4 seconds (Wetherbee and Andersen 1992). The transitory food basket of chrysomonads is formed by the dynamic interaction of flagellar root microtubules (Fig. 5; Andersen 1990, Moestrup and Andersen 1991). The so-called R_3 root typical for many chrysomonads consists of 6 microtubules, labeled *a* to *f*. The *f* microtubule of *Epipyxis pulchra* has been shown to slide up, thus increasing its loop under the short

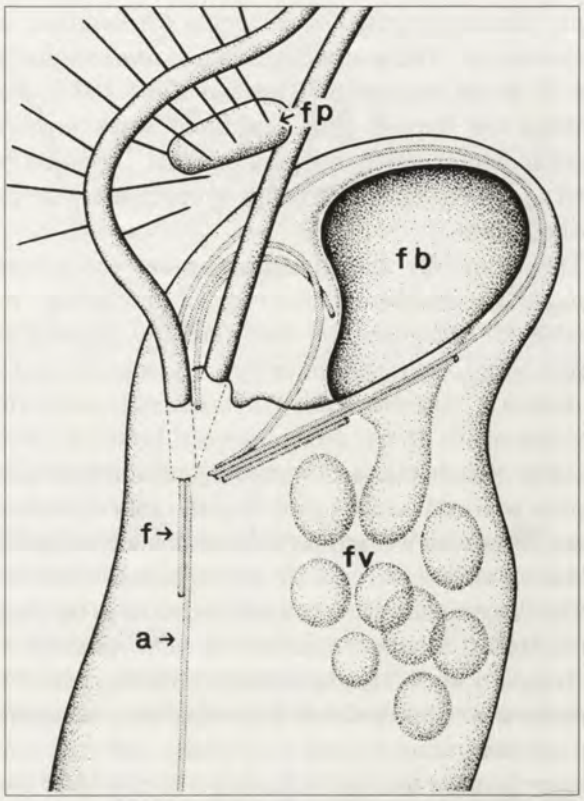

Fig. 5. Cup-like feeding apparatus of the chrysomonad *Epipyxis pulchra*. Flagellar root microtubules serve to extend the transitory food basket (fb) to engulf the food particles (fp). a – a-tubule, f – f-tubule, fv – food vacuoles. (After Andersen and Wetherbee 1992)

flagellum and enlarging the rim of the cup (Andersen and Wetherbee 1992). The *a* tubule forms at least part of the inner loop. Probably, the surface of the cup enlarges and its depth increases by fusion with two types of vesicles present in that area. Closure of the feeding cup is speculated to be mediated by possibly contractile striated fibers accompanying the *a* tubule and by periodically striated material coating the *a* and *f* tubules. The feeding cup with the internalized particle retracts, moving back to the cell body within 5-15 seconds. The food vacuole remains near the cell apex. Eventually, undigested material is expelled.

Bicoeca maris is a lorica-bearing, phagotrophic organism (Moestrup and Thomsen 1976). Bacteria which are moved close to the buccal cavity by action of the front flagellum or otherwise are ingested. The buccal cavity opens towards the exterior through a duct being supported by a complex microtubular apparatus. Moestrup and Thomsen (1976) think that the pseudopodium which was reported to take part in food uptake by Boucaud-Camou (1965) corresponds to the wall of the buccal cavity. In the loricate *Bicoeca lacustris* and *B. kepneri* the homologue of the buccal cavity, respec-

tively its walls, is a long elastic lip (Mignot 1974). Probably acting together with the front flagellum the lip directs the prey into depressions in the cell surface where phagocytosis starts.

Formation of pseudopodial structures for food catching and ingestion is used by a couple of chrysomonad species. *Monas*, e.g., forms a collar at the apical pole to entrap food particles which are swept towards the cell by the action of the long flagellum (Pringsheim 1963). The sessile *Brehmiella chrysohydra* may lose its flagella and catch prey by the formation of tentacle-like pseudopods at its apical pole. Fine, anastomosing pseudopods emerging apically are used by *Derepyxis* and *Dinobryon* to filter out food. In *Chrysarachnion* and similar forms digestion takes place either extracellularly within such a rhizopodial veil or particles are ingested by retraction of the pseudopods.

Choanoflagellida

The choanoflagellates are colorless protozoa living in marine, brackish or freshwater as single or colonial forms. Their most distinctive characters are an anteriorly directed flagellum surrounded by a ring of microvilli, the so-called collar (Fig. 6). Many species possess cell coverings such as relatively simple, close-fitting sheaths or elaborate basket-like loricas composed of ribs of silica. Species are either sedentary or planktonic. The collar tentacles filter out potential food particles such as bacteria, cyanobacteria and detritus from water currents created by base-to-tip undulations of the flagellum. In *Codosiga botrytis* the water flow is accordingly directed towards the flagellar tip (Sleigh 1964). In contrast, a water flow opposite to the propagation of flagellar waves is reported, for instance, for the chrysomonad *Monas* (= *Spumella*) and may be due to flagellar mastigonemes (Sleigh 1964). Food particles filtered from the water by the collar adhere to the mucous covering of the tentacles (Leadbeater and Morton 1974, Laval 1971). The outer collar surface seems to be selectively sticky to the prey since the remainder of the plasmalemma stays free of the mucus (Leadbeater 1983). The precise method by which the particles are then transported towards the base of the collar has been discussed controversially. What is proven so far is that linguiform pseudopods arise at its base. According to Leadbeater (1983) the pseudopods advance towards trapped food particles by moving up the outside of the collar. They function unlike the pseudopods of amoeba but totally encircle the prey and fuse

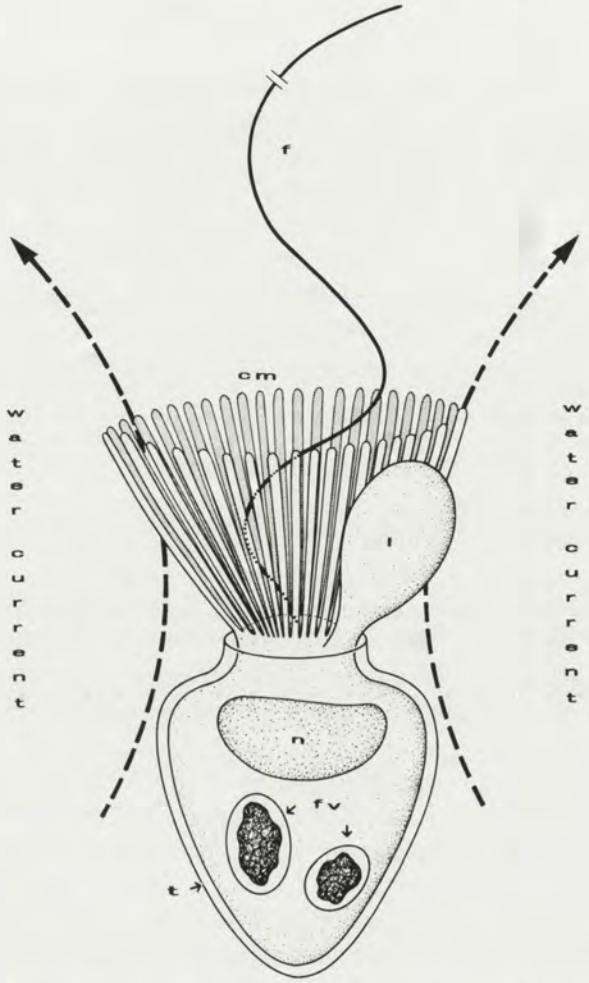

Fig. 6. Feeding choanoflagellate *Salpingoeca pelagica*. A lobopod (l) arises at the base of the collar microvilli (cm) entrapping food which has been filtered from the medium. f – flagellum, fv – food vacuole, n – nucleus, t – theca. (After Laval 1971)

with the cytoplasm of the collar almost at the site of its origin, at least in *Salpingoeca pelagica* (Laval 1971). The pseudopodium usually integrates parts of the collar. The tentacles may offer a means of support and guidance to the advancing pseudopodium (Leadbeater and Morton 1974). The trapped particles pass into the cell body at some point of the base of the collar, mostly between the protoplast and the lorica. Ingestion does not take place between the tentacles and the flagellum (Manton et al. 1976). Then, the food vacuoles are withdrawn into the cytoplasm beneath the tentacles and are apparently digested at the base of the cell. The formation of multilamellar aggregates is typical for digestive vacuoles. In collared flagellates bearing a complex lorica there is the problem of how food can reach the protoplast. The lorica of *Crinolina* is not closed posteriorly thus possibly allowing entrance of food at the posterior end (Manton

et al. 1981). Other species such as *Savillea micropora* may have elaborated a deviant nutrient regime because recognizable food vacuoles were not found at all. Sessile cells, e.g. of *Choanoeca perplexa* (Leadbeater 1977), may lose the flagellum thus precluding the production of water currents for feeding. The collar tentacles of sedentary *C. perplexa* get longer and the collar is more wide-angled. Here, food particles seem to be engulfed at the inside of the collar. Contractions cause the tentacles to pivot inside and pseudopods then advance along the inner surface and ingest the particle (Leadbeater 1977).

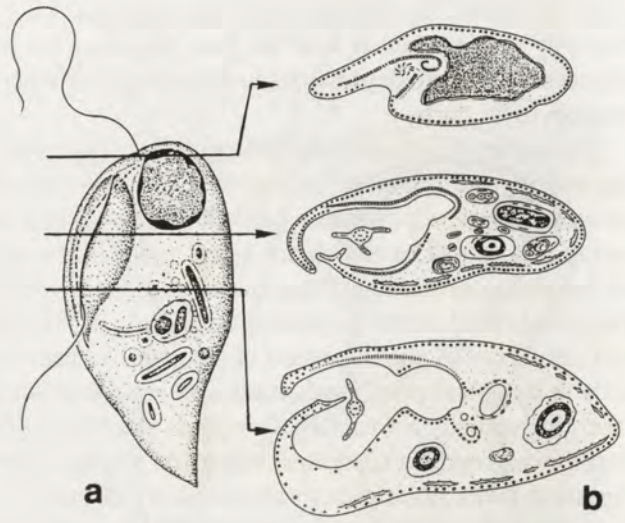

Fig. 7. Scheme of *Retortamonas* (a) with indication of section levels I, II, III (b). The recurrent flagellum with fin-like extensions passes through a groove which is bordered by cytostomal lips. (After Brugerolle and Mignot 1989)

Retortamonadida

The two retortamonadid genera *Retortamonas* and *Chilomastix* inhabit the intestine of both vertebrates and invertebrates. Retortamonads possess a large cytostome-cytopharynx complex which extends towards the posterior end of the cell (Fig. 7). The recurrent flagellum appears to generate a flow which brings food particles into the groove (Willey et al. 1988). Ribbons of microtubules and microfibrils support the edges of the groove. In contrast to the rest of the cell membrane, the groove membrane lacks subpellicular microtubules. The phagotrophic apparatus of diplomonads is similarly organized. In *Chilomastix aulastomi* the buccal invagination is bordered by two lips each equipped with a fiber composed of microtubules, that is connected with microfibrillar material. The posterior part of the right fiber has a periodic, paracrystalline structure. The buccal fibers are connected to the subpellicular microtubules. The recurrent flagellum bearing two wing-like extensions runs through the lips of the ventral oral invagination. Pinocytosis and phagocytosis occur at the cytostomal area which is free of microtubules (Brugerolle 1973). *Retortamonas* is similarly organized. Large bacteria are ingested at the microtubule-free end of the buccal invagination (Brugerolle 1977).

Trichomonadida

Trichomonads are characterized by having four flagella, an axostyle, and a Golgi apparatus associated with parabasal filaments. The families Devescovinidae and Calonymphidae are intestinal symbionts of lower termites whereas the Monocercomonadidae and Trichomonadidae inhabit the intestine, urogenital and oral cavities of invertebrates and vertebrates. They generally feed by ingestion of particulate material via invagination or pseudopod formation preferentially at the posterior cell pole or by pinocytosis at the whole surface (Brugerolle 1971). According to Kulda et al. (1986) pinocytosis takes place mostly via coated pits at the plasma membrane. The derived endocytotic vesicles fuse with primary and secondary lysosomes (Nielsen 1970).

Generally, there is no cytostome-cytopharyngeal area. Recently, Aboul-Magd and Abdel-Mawla (1989) reported a constantly present deep invagination at the anterior pole of *Trichomonas vaginalis* in scanning electron microscopic studies which seems to function as a cytostome. Nutrition appears to proceed through that cytostome and through pseudopodium-like extensions which entrap material by fusion. The cytoplasm of the pseudopods is free of cell organelles but contains numerous microfilaments (Brugerolle et al. 1974a). Drugs such as cytochalasin B and colchicine inhibit phagocytosis indicating an involvement of microfilaments and microtubules, as well (Francioli et al. 1983, Juliano and Bandiera 1985). Szreter and Tymoczko (1989) confirmed uptake of bacteria by pseudopodial enclosure at the whole surface of *T. vaginalis*. According to these authors undigestible residues are defecated.

Digestive vacuoles containing bacteria were found by Ovkinnikov et al. (1975) supporting phagocytosis of particulate matter. Food vacuoles fuse with lysosomes containing acid phosphatase. Larger lysosome-like

vesicles also arising from the Golgi apparatus contain neutral hydrolases. An appreciable proteolytic activity caused by the presence of cysteine proteinases and metalloproteinases was reported for *T. tenax* (Bózner and Demes 1991). Trichomonads inhabiting the paunch of termites feed on cellulose, e.g., wood particles. *Trichomitopsis termopsidis* possesses endo-β-1,4-glucanase and cellobiase activity to digest cellulose. The resulting glucose is glycolytically converted into pyruvate which is then fermented anaerobically to CO_2, H_2 and acetate by hydrogenosomes. Hydrolytic activity was also detected against xylan, starch and protein but not chitin (Odelson and Breznak 1985). In different *Trichomonas* species the presence of deoxyribonuclease, amylase and hyaluronidase was reported (Ryley 1967).

Oxymonadida

Oxymonads are intestinal parasites of insects. They comprise one or more karyomastigonts, each containing four flagella arranged in two pairs and one nucleus. The axial rods (axostyles) are motile in some species. Mitochondria and an obvious Golgi apparatus are absent. The small forms are reported to live osmotrophically, e.g. *Opisthomitus avicularis* (Mannesmann 1969). *Pyrsonympha vertens* and *Microrhopalodina inflata* ingest wood particles and bacteria without formation of pseudopods (Lavette 1973, 1975). Food is taken in by large invaginations of the cell surface (Bloodgood et al. 1974). The large food vacuoles may contain single, large (2-20 μm long) pieces of wood or enclose gut fluid containing bacteria, wood and debris. Furthermore, a vesicular and tubular kind of pinocytosis are reported to function in rectal fluid intake. Acid phosphatase was demonstrated in digestive vacuoles of *P. vertens* and *Dinenympha gracilis* (Lavette 1970). Digestion residues seem to be defecated at least in *Monocercomonoides* (Brugerolle and Joyon 1973).

Diplomonadida

Diplomonads occur in the alimentary tracts of animal hosts or are free-living in organically rich waters. They are characterized by a two-fold symmetry caused by two opposing karyomastigonts. The aerotolerant anaerobes lack mitochondria, microbodies and a Golgi apparatus. Brugerolle (1975a) outlined an evolutionary scheme depicting a reduction of cytostomes, i.e., flagellar pockets parallel to the flagellates passing from free-living genera to parasitic ones (*Trepomonas* - *Hexamita* - *Spironucleus* - *Octomitus* - *Giardia*). Corresponding to cytostomal reduction nutrition strategies changed from phagocytosis of particulate matter through the flagellar pockets at the posterior pole to absorption of soluble food (Fig. 8).

Treponema agilis possesses two well developed oral grooves which run for almost the complete length of the body (Eyden and Vickerman 1975). Each groove begins as a slight depression at the anterior pole and terminates as a narrow channel at the posterior extremity. The posterior channels of the two grooves seem to be continuous. The membrane of grooves and channels is asymmetric, i.e., it possesses a relatively thin outer lamina with reduced electron-density compared to the symmetric, thick other membranes. It seems to be densely covered with acid carbohydrate groups. Three rapidly beating oral flagella in each of the two grooves produce feeding currents, whereas the action of two longer locomotor flagella is of no importance for feeding. Food particles coming into contact with the groove surface are ingested into food vacuoles. The food vacuoles circulate in the cytoplasm in a dual cyclosis. Movement is saltatory and most active in the cytoplasm beneath the oral grooves and the region of the nucleus. The bidirectional pathways of cyclosis coincide with the pathways of microtubule ribbons which arise close to the basal bodies. The membrane of the food vacuoles has evaginations which are bristled on their cytoplasmic side (Eyden and Vickerman 1975). *T. agilis* ejects indigestible food particles at the same site as the contents of the contractile vacuole (Eyden and Vickerman 1975).

The situation is similar in the parasitic genus *Enteromonas* which belongs to the suborder Enteromonadina, possessing only a single karyomastigont (Brugerolle 1975b). A recurrent flagellum is lodged in a large ventral depression (oral invagination). Two lips which are equipped with microtubules and microfibrils form the border of the oral invagination. Bacteria are phagocytozed in its bottom part between the two distended lips. Indigestible residues are ejected.

Octomitus does not form an oral apparatus. Here, endocytosis occurs all over the body surface and many similarities with *Giardia* are observed (Brugerolle et al. 1974b).

Members of the parasitic genus *Giardia* adhere to the intestinal brush border of their vertebrate host by means of a ventral adhesive disc. Pinocytotic vesicles bring in nutrients through this sucking disc. The action of two

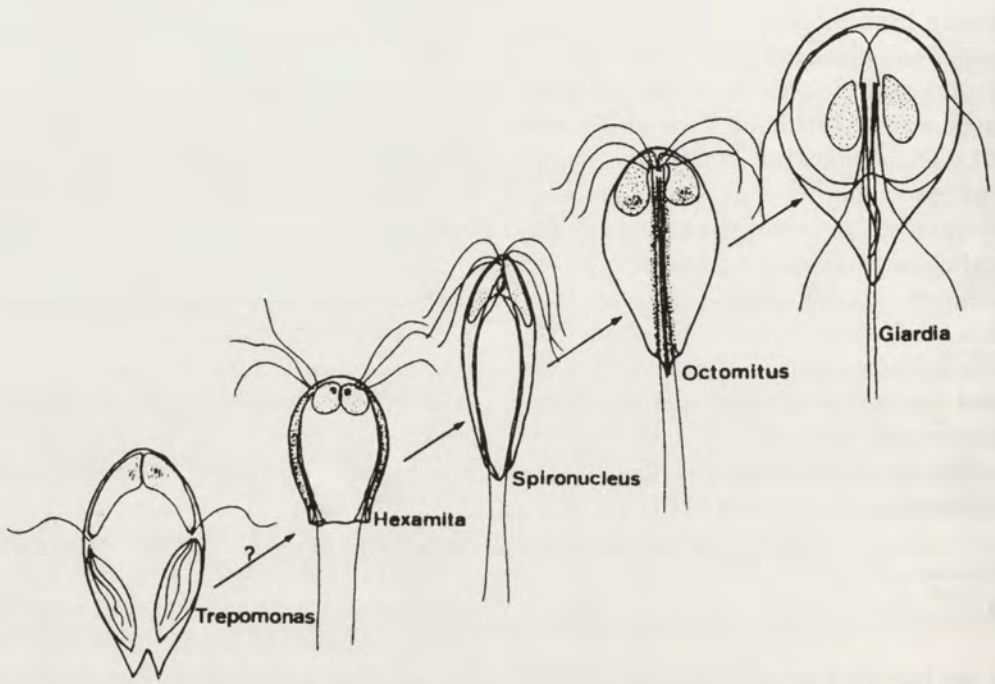

Fig. 8. Evolutionary scheme of the Diplomonadida. Compared to free-living genera the parasitic ones gradually reduced their cytostomes and their locomotory activity. (After Brugerolle 1975a)

flagella emerging from the posterior margin of the disc is thought to generate suction pressure (Vickerman 1982). Besides pinocytosis phagocytosis of rod-like bacteria being attached mainly to the dorsal region was found in *G. duodenalis* (Sogayar and Gregorio 1989). The bacteria were always in phagocytotic vacuoles and were distributed throughout the cytoplasm. They did not show any sign of degradation typical for intracellular digestion.

Giardia lamblia contains lysosome-like vesicles which are adjacent to the plasma membrane except in the region of the disc. Under experimental conditions they can accumulate ferritin and give a positive cytochemical reaction for acid phosphatase. Reaction product was also found in the endoplasmic reticulum and the nuclear envelope (Feely and Dyer 1987). Jarroll et al. (1989) detected activities of several hydrolases in an isolated lysosome-like particle population but most carbohydrate splitting hydrolases seemed to be missing. Since *G. lamblia* trophozoites were successfully cultivated in vitro the knowledge of the metabolism and the biochemistry has multiplied. Ethanol, acetate, and CO_2 are aerobically and anaerobically produced from glucose. This means that oxidation is incomplete. Metabolism starts with a glycolytic pathway. In the presence of oxygen *Giardia* respires by a flavin, iron-sulfur protein-mediated electrontransport system. The cytochrome system and the Krebs cycle are absent. Energy is produced via substrate level phosphorylation. De novo synthesis of purine and pyrimidine seem to be absent and also lipids are preferentially taken in preformed (Jarroll et al. 1989).

Hypermastigida

Hypermastigid flagellates inhabit the paunch of lower termites, cockroaches and woodroaches (Honigberg 1970). They possess numerous flagella, an elaborated Golgi apparatus, an axostyle, and hydrogenosomes instead of mitochondria. The xylophagous insects depend on their symbionts which are responsible for cellulose digestion. Although coexisting oxymonads and trichomonads also feed on cellulose, only the large hypermastigotes seem to be essential for food digestion. Food particles are not ingested at the densely and regularly flagellated body portions but at the less structured, deformable posterior region. The plasma membrane is apparently somewhat sticky for food particles coming into contact with it (Cleveland 1925). Yamaoka (1979) stated a decline in cellulose ingestion after treatment of *Trichonympha* with proteolytic enzymes. An intact filamentous surface coat seemed to be essential for food trapping and selective ingestion of cellulose. Other solid

materials were not ingested. Descriptions of the process of endocytosis vary between different authors and different species. In *Trichonympha* food intake by invagination (Cleveland 1925) was also described as ingestion by 'pseudopodial' methods (Swezy 1923). Emik (1941) found two types of pseudopodial, i.e., amoeboid processes depending on food particle size. Large pieces or aggregates of small particles were ingested amoeboidly by the pear-shaped organisms. Fine particles were taken in by bell-shaped invaginations which created a posterior cavity into which the food was drawn and engulfed by closing the cavity. According to the author the true picture of ingestion may be a modification since the characteristic shapes seemed to be induced by experimental conditions. *Joenia annectens* glues wood particles to the body zone which is covered with bacteria and pinches the particles off into the cytoplasm without forming pseudopods (Lavette 1966, 1967). Thus food vacuoles are often internally covered by attached bacteria which are a supplement for nutrition.

The end products of cellulose and hemicellulose digestion are well known. CO_2, H_2, and mainly acetate as an organic acid are produced by anaerobic fermentation and are released into the medium (Breznak 1982). The process of enzymatic degradation and the origin of the enzymes is still not totally elucidated. Several types of cellulases are involved (endo-, exoglucanases, cellobiases). Endosymbiotic bacteria could contribute to digestive enzyme production, though, e.g., bacteria-free cultured *Trichonympha* could survive without endosymbionts (Yamin 1981). Acid phosphatase was detected in the Golgi apparatus (Lavette 1967). The extent of lignin degradation is yet controversial (Breznak 1982).

Besides phagocytosis an intense pinocytotic activity was reported, e.g., for *J. annectens* and *J. duboscqui* (Hollande and Valentin 1969). At the level of the Golgi apparatus thousands of tubules with a mean length of 1 µm and a diameter of 60 nm lie in the ectoplasm, perpendicular to the plasma membrane. Active pinocytosis could be followed by addition of the marker substance thorotrast. Coated vesicles pinch off and were apparently charged with electron dense material by ER cisternae.

According to Swezy (1923) particles may be ejected at the posterior pole of xylophagous flagellates whereas Lavette (1967) never observed defecating flagellates. *J. annectens* was reported to get rid of multivesicular, residual bodies via ejection during cell division (Hollande and Valentin 1969).

Kinetoplastida

The Kinetoplastida are characterized by a single mitochondrium with one or more kinetoplasts and one or two flagella with a paraxial rod. The suborder Bodonina includes mostly genera with free-living species being main consumers of bacteria in aquatic and soil systems (*Bodo, Rhynchomonas*) but also endo- and ectoparasitic forms (*Cryptobia, Trypanoplasma, Ichthyobodo*). Most species efficiently use a cytostome-cytopharynx complex in coaction with a sweeping flagellum to trap bacteria. Mastigonemes can support the generation of feeding currents, e.g., in *B. saltans*. Bodonids feed while creeping along or being attached to a substratum and/or while swimming freely. Thus attached and suspended food particles can be caught, as well (Mitchell et al. 1988). *B. saltans* showed a modified preference for different species of bacteria. The motility or secretory activity of the bacteria may influence the efficiency of their utilization. A possible response to bacterial attractants remained obscure. *B. crassus* exhibits an unusual feeding habit. It is reported to consume exclusively cells of *Synura* (Chrysophyceae). A first contact is done by entangling of the flagella of *B. crassus* with the flagella of a *Synura* colony. The closer contact stimulates pseudopodial development in the flagellar insertion region of *Bodo*. The pseudopods then enclose a *Synura* cell, pull it free from the colony and engulf it into the cytoplasm. Several prey cells are ingested and gradually digested (Nicholls 1987). *Colponema loxodes* also does not possess an oral apparatus but ingests prey cells by submerging them into the body via formation of a deepening depression (Mignot and Brugerolle 1975a). The cytoplasm underlying the simple membrane is conspicuously clear at the site of ingestion and contains microfibrils, resembling pseudopod structure of amoebae. Toxicysts were proposed to deliver digestive enzymes for they were found in close proximity and vesicles resembling primary lysosomes were not present at all. The parasitic *Ichthyobodo* attaches to an epithelial cell of its host, a freshwater fish, and then penetrates it with a rostrum which contains a buccal invagination (Joyon and Lom 1969). Extracellular digestion of host cell material with subsequent absorption of molecules was proposed by the authors for no signs of an endocytotic process were found.

The bodonid feeding apparatus generally consists of a pocket (oral invagination) originating anteriorly adjacent to the flagellar opening. Interconnected microtubules running along its length support the pock-

et. The number and arrangement of the microtubules vary in the different species, just as the structure of the area surrounding the buccal cavity. Mostly, a cytoplasmic ridge (preoral ridge) passes from the flagellar pocket into the buccal cavity. The ridge is also supported by the buccal microtubules which further run along the flagellar pocket, arising close to the basal bodies. The cytoplasmic ridge represents an integral part of the feeding apparatus. Together with the mobile, anterolateral protruding rostrum the preoral ridge may play an active role in seizing and taking in the prey (Nohýnková 1984). In *Rhynchomonas* the edges of the buccal cavity extend forming a mobile proboscis (Swale 1973). Food organisms are tested in the lumen of the proboscis and if they are ingested the proboscis becomes short and wide and sucks the food into a membrane-limited lumen. The rows of microtubules which surround the lumen are continuous with the cytopharyngeal microtubules. Burzell (1973) speculated that the inner ring of microtubules lying close to the lumen of the proboscis may move to the outer microtubular ring during the process of food ingestion thus widening the lumen. A comparable mechanism was proposed for suctorian food intake (Bardele 1972). Widening of the buccal invagination for food ingestion was described for several species, for instance *B. designis*. In *B. designis* and *B. curvifilus* a compact, prismatic microtubular rod is associated with the oral invagination which is speculated to be involved in the ingestion process (Eyden 1977). Displacement of the rod-organelle as a whole or sliding of its microtubular rows may cause an extension of the oral apparatus. Connections of some of the typical supporting microtubules with the membrane of the oral invagination may also promote membrane movement during ingestion. The oral apparatus of the colonial flagellate *Cephalothamnium cyclopum* consists of a funnel-shaped buccal cavity (Hitchen 1974). It is supported by various fibrillar systems such as helical and longitudinal microtubules and striated fibers. The free flagellum is important in food gathering. The origin of the abundant oral vesicles is uncertain. It is suggested that they are in the process of exocytosis rather than pinocytosis. Digestive enzymes may be mixed with the food prior to endocytosis. Another purpose could be to contribute membrane material to the enlarging lining of the cytostome. The buccal microtubules have arm-like projections toward the membrane, implicating a mechanochemical activity in the transport of membrane material down the oral apparatus.

Cryptobia sp. parasitizing the spermatheca of the land snail *Triadopsis multilineata* possesses an elaborate cytostome-cytopharynx complex (Current 1980). Microtubules support a preoral ridge that emerges from the right-ventral portion of the flagellar pocket and passes into the oral invagination. The oral invagination is associated with 8 parallel microtubules. 5 are aligned in an arc on the left side, the remaining 3 lie dorsally. The microtubules are not connected to the feeding channel membrane. Small food vacuoles are scattered throughout the cytoplasm whereas large ones are located primarily in the posterior region of the cell.

The parasitic suborder Trypanosomatina is of considerable economic and medical importance. During the passage through different developmental stages and hosts polymorphic forms with changed metabolism and nutrition arise. The plasmalemma is underlain by pellicular microtubules except for the flagellar pocket where the flagellum is anchored. The position of the flagellar base in relation to the nucleus accounts for the various morphological forms, i.e., a-, pro-, epi- and trypomastigotes. The trypanosomes of mammals fall into two groups. The Stercoraria complete their life cycle mainly in the hindgut of insects and are transmitted via feces, whereas the Salivaria get infective in the salivary glands of insects and enter the mammalian host during blood-sucking.

Endocytotic intake of food is restricted to the membrane of the flagellar pocket in the salivarian trypanosomes which lack an additional cytostome-cytopharynx complex (Langreth and Balber 1975). A cytostome-cytopharynx is also lacking in *Leishmania, Phytomonas* and other monogenetic trypanosomes except for *Crithidia*. In thin sections of *T. brucei gambiense* an electron-dense ring around the neck of the flagellar pocket was visible which might regulate the entrance of blood constituents into the pocket (Yoshikawa et al. 1990). Initial binding of material may happen all over the body surface. In this case it is transported to the sites of endocytosis. Freeze-fracture studies of *T. brucei gambiense* revealed depressions on the flagellar pocket membrane rich in intramembraneous particles which may correspond to the sites of pinocytosis (Yoshikawa et al. 1990). Coated vesicles filled, e.g., with tracer proteins have been found arising from the pocket membrane of bloodstream forms of *Trypanosoma brucei*. The vesicles become continuous with a complex tubular membrane system in which ingested material gets concentrated and digested (Langreth and

Balber 1975). The coated vesicles differ from clathrin-coated vesicles from other eukaryotes in being larger (100-150 nm) and containing an inner coat of electron-dense material in addition to the external coat (Shapiro and Webster 1989). The internal coat is suggested to be the parasite's variant surface glycoprotein coat. A minor protein component comigrating with mammalian clathrin in SDS-PAGE did not show serological cross-reactivity with clathrin. Coppens et al. (1987) studied the uptake of various host plasma proteins by the same parasite and found two different mechanisms of endocytosis. Total plasma protein, serum albumin and ferritin were taken up by endocytosis of fluid with a low clearance rate of the medium implying simple emptying of the flagellar pocket by formation of endocytotic vesicles (about 250 per hour). In contrast, low-density lipoprotein (LDL) and transferrin were caught by selective receptor-mediated and Ca^{2+}-dependent processes, probably via coated vesicles, with a clearance of two to three orders of magnitude higher. It is likely that the LDL particles have an important function in making cholesterol available for the trypanosome.

Some stercorarian trypanosomes and the monogenetic *Crithidia* possess a cytostome-cytopharynx complex opening near the flagellar pocket (*T. mega, T. danilewskyi*) or into it (*T. raiae, T. cruzi, C. fasciculata*). A convincing cytostomal complex has not yet been demonstrated in any bloodstream stage of a trypanosome, except for the fish trypanosome *T. danilewskyi* (Lom et al. 1980). Per definition a cytostomal complex is reinforced by microtubules and located above the kinetoplast (Lom et al. 1980). The extension of the flagellar pocket of the haematozoic form of *T. avium* should thus not be named cytostomal complex. In *T. raiae* there is a group of 5 to 6 microtubules running along the length of the buccal invagination (Preston 1969). They, at least in part, originate in the flagellar pocket and run a short distance beneath the cell surface before coursing into the cytoplasm around the buccal opening. Delicate fibers connect the microtubules with the membrane of the buccal invagination in *T. raiae, T. cruzi* and *C. conorhini*. Milder and Deane (1969) suggested that the entrance of the organelle may be opened and closed by the action of the connected microtubular systems. The microtubules also appear to define the path taken by the pinocytotic vesicles through the cytoplasm (Brooker 1971).

In some trypanosomatids at least two pathways exist for the ingestion of extracellular material. Ferritin is included both in coated vesicles pinched off from the flagellar pocket membrane and in larger smooth vesicles which arise from the oral invagination. The necessity for two pathways is not clear but the possibility of selective endocytosis by coated vesicles may be of relevance. In *C. fasciculata* both types of vesicles fuse with multivesicular bodies which contain acid phosphatase activity and which are thought to be important for intracellular digestion (Brooker 1971). The intracellular stage of *T. cruzi* does not only feed by pinocytosis and diffusion across the plasmalemma but additionally by ingestion of host cytoplasm via phagocytosis through an oral apparatus (Meyer and De Souza 1973).

Besides endocytotic uptake of nutrients, mediated active transport is of particular importance in parasitic protozoa. Glucose and leucine enter *T. lewisi* by active transport, while K^+ enters by diffusion (Schraw and Vaughan 1979).

Acid phosphatase activity has been demonstrated cytochemically in various culture and bloodstream stages and in intracellular trypanosomatids. Bloodstream stages of *T. brucei* possess a cytochemically detectable phosphatase activity within the flagellar pocket. Released enzymes may serve to predigest the substrate (Langreth and Balber 1975). *Leishmania donovani* secretes an acid phosphatase which could be shown to be a phosphorylated glycoprotein (Lovelace et al. 1986, Lovelace and Gottlieb 1987). Furthermore, the parasite has low levels of most hydrolytic enzymes with the exception of non-specific acid phosphomonoesterase activity, which is located in promastigotes on the external surface and within intracellular vacuoles (Lovelace et al. 1986). According to Vickerman and Preston (1976) lysosome populations of trypanosomatids seem to be heterogeneous, i.e., compartments with different enzyme compositions assigned for special tasks exist. Various enzymes have been recorded. In *T. theileri* and *T. melophagium*, e.g., β-glucuronidase, lipase and non-specific carboxylic esterase were found, in *Crithidia luciliae* four hydrolytic enzymes were assayed (β-glycerophosphatase, β-fructofuranosidase, proteinase, deoxyribonuclease) (Herbert 1965, Eeckhout 1970).

Trypanosomes seem to eject end products of digestion and autophagy into the flagellar pocket. Multivesicular bodies, e.g., do not in general accumulate in large numbers in the cytoplasm and debris is often found in the pocket. Langreth and Balber (1975) showed a micrograph of whorls of membranes and other debris which appeared to be fixed in the process of being extruded.

Opalinida

The opalines are multinuclear protists lacking an oral apparatus which are totally covered by short flagella. Patterson (1985) presented an account of the confusing opalinid classification and phylogeny. Living in the rectum of cold-blooded vertebrates as endocommensals their environment is rich in nutrients. Food uptake involves the pinching off of small endocytotic vesicles from the cell membrane at the base of the pellicular folds. The newly formed vesicles are aligned by the lattice of microfilaments into rows transverse to the pellicular folds (Wessenberg 1978). Spherical coated vesicles and more irregular formed, smooth vesicles arise in *Cepedea dimidiata* and other genera which might bring different types of nutrients into the cell (Noirot-Timothée 1966).

With the aid of ThO_2 as a tracer the process of endocytosis, digestion and exocytosis could be followed in *Opalina ranarum* (Fig. 9; Münch 1970). Endocytosis was not enhanced by accumulation of tracer to the mucopolysaccharide coat of the plasma membrane. The small endocytotic vacuoles fuse into large food vacuoles. Digestive vacuoles showed cytoplasmic, microvilli-like projections into their interior and small vesicles being attached to the periphery. Münch (1970) proposed the latter to be Golgi vesicles delivering digestive enzymes and membrane material. Acid phosphatase which is generally associated with the digestion of material was found in food vacuoles, lysosome-like vesicles and the Golgi complex (Sergejeva 1969). Digestive vacuoles do not appear to circulate in the cytoplasm. Indigestible residues are concentrated and removed by *Opalina ranarum* via exocytosis (Münch 1970).

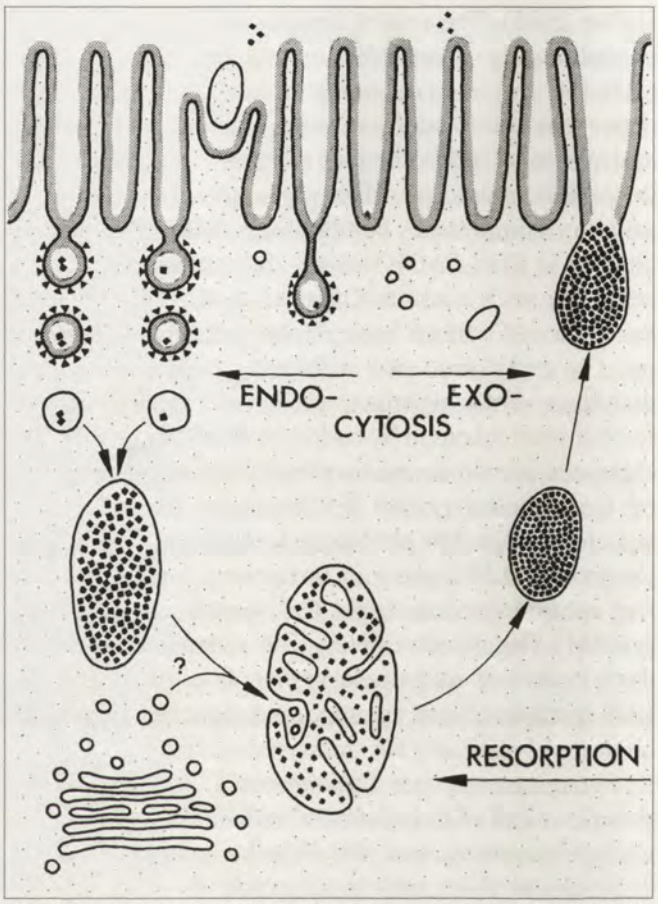

Fig. 9. Cytotic processes in *Opalina ranarum*. (After Münch 1970)

Miscellaneous

In the following diverse phagotrophic protozoa are treated which are either insufficiently classified (incertae sedis) or belong to a systematic group of which only scarce information was available.

The marine, quadriflagellate *Percolomonas cosmopolitus* is placed incertae sedis in the Sarcodina, class Heterolobosea (Fenchel and Patterson 1986). The organism feeds when it is attached to the substratum by the tip of the long flagellum. By this, the dorsal surface comes into contact with the substratum so that the ventral surface with a long groove is exposed to the free water, facilitating access of food organisms. The free short flagella, lying against one another to form a narrow sheet beat synchronously. Their action creates a backward directed current of water which carries particles to the posterior end of the groove where they may be ingested.

Cercomonas (= *Cercobodo*), as well, comes into contact with the substrate (Mignot and Brugerolle 1975b). It glides with a recurrent flagellum. Parallel, undulating pseudopods arise laterally while at the posterior pole lobes are extended which internalize prey. *Cercomonas* has many features of chrysomonads. The colorless *Apusomonas* and *Amastigomonas* for which a new order Apusomonadida is proposed behave similarly (Karpov and Myl'nikov 1989). They crawl across the substrate while pseudopods are extended from the edges of a ventral groove to capture and engulf food.

Another type of pseudopods is formed by the colorless flagellate *Thaumatomonas lauterboni* (Karpov and Makarenkova 1989). Within several seconds long, ramified filopods grow from the ventral groove and entangle the food particles. A population of small ventral vesicles serve as a membrane reservoir for the quick

formation of the vast surface of the filopods. The vesicles are of endocytotic origin rather than being Golgi derivatives. Coated vesicles are pinched off from the dorsal surface and from the flagellar pocket and are further transported to the ventral part of the cell losing their clathrin coat. There is a constant ventral storage of small vesicles.

The newly discovered flagellate *Pirsonia guinardiae* is a parasite on the marine diatom *Guinardia flaccida* (Schnepf et al. 1990). It exhibits a quite unusual mode of food uptake. It attaches to the valvae or the girdle region of its host cell and forms a process near the apical cell pole which pierces the weakly silicified cell wall of the diatom. Inside, the process develops into a so-called trophosome, consisting of a proximal digestion vacuole and distal pseudopods which phagocytoze host cytoplasm. The main part of the body (auxosome) remains outside the host cell. The diatom reacts to the invasion by creating a stream of cytoplasm including chloroplasts toward the infection site. The cytoplasm concentrates there and is partly ingested by pseudopodium-like outgrowths of the developing throphosome which penetrate into the host protoplast. The phagocytotic vesicles migrate to the proximal digestion vacuole of *Pirsonia* and fuse with it. No vacuoles appear in the auxosome. The auxosome develops in a special manner, multiplies and detaches from the host cell whereas only parts of the trophosome are retracted and the rest disintegrates. It remains as a residual body containing indigestible residues between the recovered plasmalemma and the cell wall of the diatom.

The genus *Katablepharis/Kathablepharis* possesses a feeding apparatus at the anterior cell pole (Lee et al. 1991, Vørs 1992). It consists of a raised ridge surrounding a central depression, the cytostome. The anterior ends of an inner and outer array of microtubules similar to these of the Suctoria support the raised ridge. They function in the uptake of prey organisms such as small flagellates and bacteria. Membrane cisterns occur close to the cytostome and two types of vesicles, one of them with electron-dense contents, are associated with the microtubular arrays. The related genus *Leucocryptos* is similarly organized possessing a small slitlike or rounded opening, the cytostome, at the periphery supported by one or more ring-shaped, tubular vesicles, and associated with the anterior ends of bundles of pellicular microtubules (Vørs 1992). *L. marina* seems to feed by 'sucking' the contents of a prey.

Stephanopogon species resemble ciliates in moving by means of several rows of flagella, in possessing a conspicuous cytostomal-cytopharyngeal apparatus and in behaving like benthic marine gymnostomes. But, besides their homokaryotic status, they have further non-ciliate characteristics that support their relationship to flagellates (Lipscomb and Corliss 1982, Patterson and Brugerolle 1988). *Stephanopogon* consumes bacteria, diatoms, flagellates, yeast cells, and organic detritus by engulfment via an oral apparatus. The oral apparatus lies at the anterior end of the cell (Patterson and Brugerolle 1988). It is bordered by hyaline, non-flagellated lips from between which a flat oral invagination runs into the body. Oral and cortical microtubules arise from a fibrous structure lying in the swollen anterior part of the lips instead of arising from kinetosomes as do those of the ciliates. Two categories of oral microtubules exist. One forms rods containing interconnected microtubules with an unusual quadratic packing. The single rods are not rigidly connected and may flex relative to each other. They support the oral invagination. The second type of oral microtubules is single, evenly spaced and supports a thin, fenestrated sheet of electron-dense material (the oral sheet) (Patterson and Brugerolle 1988). The area of the oral apparatus is rich in phagoplasmic vesicles (Lipscomb and Corliss 1982).

Pteridomonas is a stalked helioflagellate, i.e., a colorless flagellate which resembles heliozoa in having stiff arms which radiate from the body (Patterson and Fenchel 1985). The single, anterior flagellum of *P. danica* is surrounded by about 12 fine arms. Regular undulations of the flagellum in a single plane pull a current of water down and through the arms which trap bacteria and other small particles before ingestion. Actively feeding cells may fuse together to create multinucleated organisms, as do many heliozoa.

In the parasitic flagellates *Proteromonas* and *Karotomorpha* (order Proteromonadida) the phenomenon of phagocytosis has not been observed but, however, an intense pinocytotic activity was evident (Brugerolle and Joyon 1975). Pinocytotic vesicles containing thorium particles added to the medium are abundant in the peripheral cytoplasm of the posterior cell region. The vesicles seem to coalesce to larger digestive vacuoles. Acid phosphatase activity was apparent in these digestive vacuoles and in the Golgi apparatus plus its derivatives. Indigestible residues are ejected via exocytosis (Brugerolle and Joyon 1975).

A number of species of the flagellate order Prymnesiida (= Haptophyceae) have been shown to be mixotroph, ingesting particulate matter especially at low light intensities (Jones et al. 1993, Parke and Manton

1962). In *Chrysochromulina hirta* it has recently been shown that the haptonema is involved in the acquisition of food particles (Kawachi et al. 1991). Food becomes aggregated on the haptonema and is deposited by it at the posterior cell pole where it is ingested. In *C. brevifilum* not the haptonema but the action of the two flagella seems to move prey particles to the sensitive non-flagellar pole (Jones et al. 1993). The formed food vacuole enclosing, e.g., ingested small green flagellates remains at the posterior cell pole. The flagellates are well digested within one hour.

Conclusions

Internalization of particulate and dissolved nutrients by endocytosis is not only typical of colorless flagellates but is also a widespread phenomenon among green species. Since an efficient food uptake is essential for the survival of organisms, different environmental conditions supported the development of a variety of ingestion mechanisms. Thus, also within the same taxonomic group of flagellates different nutritional strategies have been realized. Many of the different feeding devices seem to have been developed independently during evolution since also the flagellates themselves are of polyphyletic origin. The molecular mechanisms concerning food vacuole growth, its pinching off and transport in the cytoplasm and the details of the digestion cycle are known to date rather incompletely for flagellates.

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Morphologie et Ultrastructure du Cilié *Condylostomides grolieri* gen. n., sp. n. (Ciliophora: Heterotrichida)

Morphology and Ultrastructure of the Ciliate *Condylostomides grolieri* gen. n., sp. n. (Ciliophora: Heterotrichida)

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Resumé. *Condylostomides grolieri* gen. n., sp. n., Cilié non contractile, vivant dans l'eau d'une source hydrominérale riche en carbonate de calcium, a une ultrastructure somatique typique de celle d'un Hétérotriche à base de dicinétides. Le cinétosome antérieur de chaque dicinétide est cilié et associé à un rideau de 13-15 microtubules transverses, doublé de 2 microtubules dans le secteur du triplet 5. Le cinétosome postérieur est nu, associé à 2 microtubules transverses dans le secteur des triplets 4-5, une fibre striée catetodesmale dirigée sur la droite, perpendiculairement à la cinétie, dans le secteur des triplets 5-6, un rideau de microtubules postciliaires accompagné à droite d'une lame retrodesmale, à gauche d'une fibre retrodesmale à structure périodique, cette dernière représentant le caractère original du cortex de *Condylostomides*. La structure des organelles buccaux est très proche de celle des organelles de *Fabrea*, voisine de celle des organelles de *Phacodinium et Copemetopus*. Chaque organelle adoral a 3 rangées de cinétosomes. Ceux de la rangée droite ont un rideau de fibres postciliaires, ceux de la rangée gauche ont un rideau de microtubules transverses, doublé d'un microtubule supplémentaire, seul présent sur tous les autres cinétosomes de l'organelle, qui sont également porteurs de némadesmes.
L'organelle paroral est une double rangée de cinétosomes avec des rideaux de microtubules postciliaires sur les cinétosomes de la rangée externe, un microtubule transverse sur les cinétosomes de la rangée interne. Dans le trajet antérieur de la parorale, de faon irrégulière, les cinétosomes de la rangée interne peuvent être disposés perpendiculairement l'un à l'autre. En outre, la parorale est doublée dans son tiers antérieur par des segments de cinéties de 4 cinétosomes, tous porteurs de microtubules postciliaires, d'un microtubule transverse et de némadesmes.

Mots clès. Morphologie, ultrastucture, Cilié, Heterotrichida.

Summary. The non-contractile ciliate *Condylostomides grolieri* gen. n., sp. n. inhabiting calcium carbonate-mineral springs, shows a dikinetid based-somatic ultrastructure, typical for Heterotrichs. The anterior kinetosome of each dikinetid is ciliferous and is associated with a ribbon of 13-15 transverse microtubules (T1), with 2 additional microtubules (T2) at the level of the triplet 5 zone.
The posterior kinetosome is naked, associated with 2 transverse microtubules in the triplets 4-5 zone, a catedodesmal striated fibre directed towards the right, perpendicularly to the kinety in the triplets 5-6 zone, a ribbon of postciliary microtubules which is accompaned at the right by a retrodesmal strip and at the left by a retrodesmal fibre showing a periodical structure. This latter represents the original characteristic of *Condylostomides* cortex.

The structure of buccal organelles resembles that observed in *Fabrea*, *Phacodinium* and *Copemetopus*. Each adoral organelle bears three rows of kinetosomes. The right row-kinetosomes show a ribbon of postciliary fibres. The kinetosomes located on the left row have a ribbon of transverse doubled microtubules with an additional single microtubule, present on all other nematodesma bearing-kinetosomes of the organelle.

The paroral organelle consists of a double row of kinetosomes with, on the one hand, ribbons of postciliary microtubules associated with kinetosomes of the external row and, on the other hand, a transverse microtubule associated with kinetosomes of the internal row. Over the anterior course of the paroral, and through an irregular arrangement, the kinetosomes of the internal row can be perpendicular one to another. Moreover, the anterior third of the paroral is doubled by the kinetofragments of 4 kinetosomes which all bear postciliary microtubules, a transverse microtubule and nematodesma.

Key words. Morphology, ultrastructure, Ciliate, Heterotrichida.

INTRODUCTION

Les arbres moléculaires ont montré l'ancienneté du groupe des Hétérotriches (Schlegel 1991) et les grandes distances phylogénétiques entre les espèces libres de ce groupe. Or, si les observations ultrastructurales ont souligné une diversité des structures buccales, elles ont, par contre, révelé une homogénéité des structures corticales (Silva Neto et al. 1992). Il en ressort la reconnaissance générale d'un certain nombre d'ordres (Tuffrau et Puytorac - sous presse) chez les Heterotrichea, mais une étude de nouvelles espèces au microscope électronique reste nécessaire pour une comparaison ultrastructurale plus complète et plus précise. D'où ce travail sur un cilié Hétérotriche nouveau.

MATERIEL ET METHODES

C. grolieri a été récolté avec des *Caenomorpha* et *Metopus* dans l'eau d'une source hydrominérale d'Auvergne (France), riche en carbonate de calcium.

Les Ciliés ont été imprégnés par l'argent selon la technique de Tuffrau (1967a), modifiée par Grolière (1980). Pour l'observation au microscope électronique à transmission, les ciliés ont été fixés dans du glutaraldéhyde à 2,5% dans le tampon phosphate (pH = 7.2), rincés dans le même tampon, postfixés au tetroxyde d'osmium à 2% dans le tampon phosphate (pH = 7.2) les 2 fixateurs ayant une concentration finale de l'ordre de 400 miliosmoles. Après rinçage dans le tampon phosphate, les ciliés ont été déshydratés et inclus dans la gélose puis dans l'Epon 812.

Les coupes ont été contrastées par l'acétate d'uranyle et le citrate de plomb, et examinées au microscope Siemens Elminskop 1A.

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RESULTATS

Morphologie générale. Déformable, de coloration sombre, le Cilié est allongé (250- 400 x 90-140 µm), avec une large partie antérieure, obliquement tronquée. Trente à trente cinq cinéties somatiques, dont 10-11 postbuccales (Figs. 1, 2) recouvrent la cellule. Le péristome circulaire, antérieur, porte une frange adorale d'une trentaine d'organelles et, du côté droit, une frange parorale de longueur comparable à celle de l'AZM. Les organelles buccaux aboutissent à un entonnoir infundibulaire spiralé (Figs. 1, 2, 3). L'organelle paroral est formé de 2 rangées de cinétosomes cilifères, doublées dans leur parcours antérieur par 4 autres courtes rangées de 4 cinétosomes également cilifères (Figs. 1, 3, 4) formant des organelles apicaux. Un tapis de microtubules reliant les infraciliatures adorale et parorale sous-tend la cavité buccale (Figs. 1-4).

L'appareil nucléaire est formé de 3 macronoyaux sphériques (10-15 µm de diamètre) généralement isolés (Fig. 5) et d'un micronoyau. Une vacuole pulsatile est en position postérieure. Le cytoplasme est riche en ingesta (petits ciliés, diatomées) (Fig. 5).

Ultrastructure.

Cortex. Sous la membrane plasmique, une couche alvéolaire est inégalement distribuée, discontinue (Figs. 6, 7). Il n'y a pas d'épiplasme, ni de limite ecto-endoplasmique, ni de système microtubulaire apparent. Les cinéties, implantées dans les sillons limités par les crêtes ectoplasmiques, sont constituées de dicinétides obliquement disposées par rapport à l'axe des cinéties (Fig. 6). Seul cilifère, (Figs. 7, 12), le cinétosome antérieur est associé à un rideau de 13-15 microtubules transverses (T1), doublé de 2 microtubules (T2) dans le

secteur du triplet 5 (Figs. 8, 13). Le cinétosome postérieur est associé à: 2 microtubules transverses (TP) dans le secteur des triplets 4-5 (Figs. 10, 13); une fibre non striée (KN) transversalement dirigée du côté droit, dans le secteur du triplet 5-6 (Figs. 8, 9, 13); une fibre striée, postérieurement dirigée, accompagnant comme une lame rétrodesmale, le rideau de microtubules postciliaires orientés vers la partie postérieure, dans le secteur du triplet 9 (Figs. 7, 9, 13). Des organelles implantés perpendiculâirement à la membrane pourraient être des mucocystes (Figs. 6, 15, 19).

Cavité buccale. Les organelles adoraux, séparés par de larges crêtes ectoplasmiques (Figs. 11, 12, 19), sont formés chacun de 2-3 rangées de cinétosomes, la rangée antérieure étant la plus courte. Dans chaque organelle, les cinétosomes de la rangée gauche sont associés à un rideau de microtubules transverses (T1) accompagné d'une fine lame dense aux électrons, et doublé d'un microtubule complémentaire (T2). Tous les autres cinétosomes sont associés à 1 ou 2 microtubules transverses (T2). Les cinétosomes de la rangée droite sont associés à un rideau de fibres postciliaires. Les cinéties des différentes rangées sont reliées par des desmoses enrobées de matériau dense aux électrons (Figs. 19, 21). Des némadesmes sont issus de la partie proximale de tous les cinétosomes.

L'organelle paroral, de la région infundibulaire jusqu'en avant du bord antérieur du péristome est constituée de 2 rangées de cinétosomes. Dans le trajet antérieur de la rangée interne, des cinétosomes jumelés sont implantés presque perpendiculairement l'un par rapport à l'autre (Figs. 22, 24, 26). A l'extrémité antérieure, au niveau des premiers organelles adoraux se juxtaposent à la droite des 2 rangées de cinétosomes paroraux, d'autres courtes rangées de 4 cinétosomes cilifères sont reliées par des desmoses (Figs. 20, 22, 23). Dans la double rangée interne des cinétosomes, ceux qui sont le plus à gauche, reliés entre eux par un tractus microfibrillaire à noeuds, sont associés à une fibre microtubulaire transverse isolée (T), ceux qui sont le plus à droite - à un rideau de microtubules postciliaires. De tous les cinétosomes sont issus des némadesmes.

Au niveau du bourrelet cytoplasmique, la carena de Tuffrau (1967b) séparant la frange adorale d'organelles de l'organelle paroral, des vésicules pharyngiennes, allongées, aplaties, sont entassées en paquets orientés perpendiculairement à la membrane qui est renforcée de rideaux de microtubules postciliaires (Figs. 11, 12, 27).

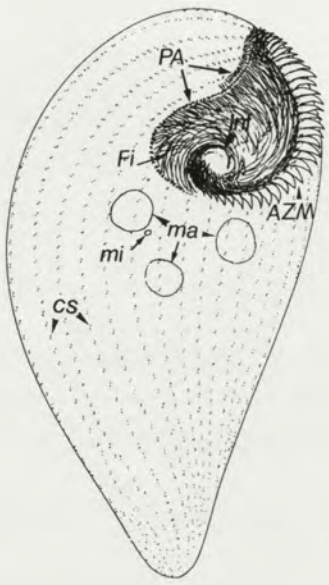


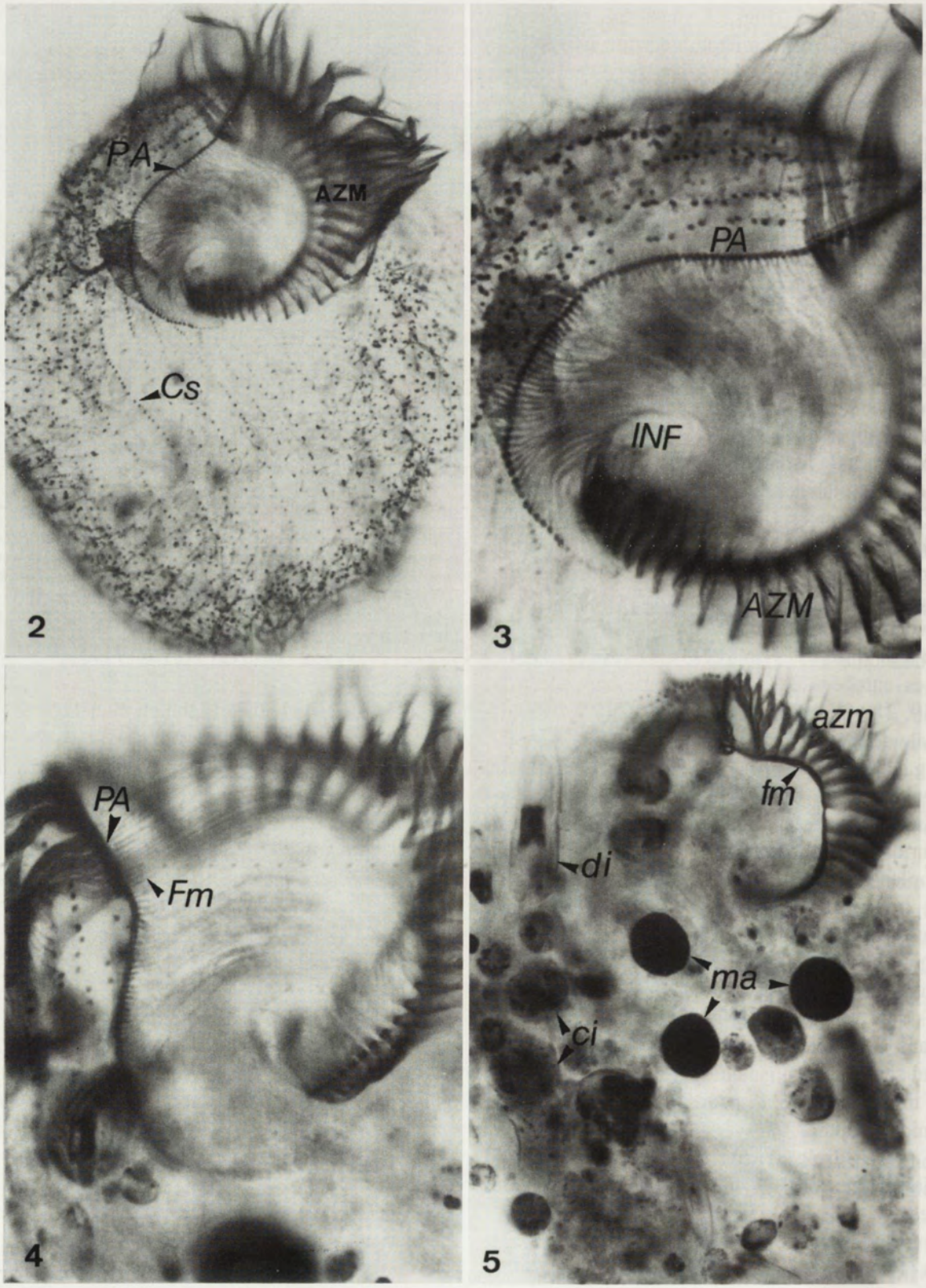
Fig. 1. Schéma général de *Condylostomides grolieri* avec les cinéties somatiques (cs) faites de dicinétides, l'appareil buccal comprenant la partie péristomienne avec organelles adoraux (AZM) et organelle paroral (PA), la partie infundibulaire (Inf.), les fibres (Fi) issues des organelles buccaux, l'appareil nucléaire (macronoyau - ma; micronoyau - mi)

Appareil nucléaire. Riche en nucléoles, les macronoyaux contiennent souvent des organismes parasites nucléés (Fig. 30). Le micronoyau est proche d'un macronoyau, dans une travée cytoplasmique (Fig. 29).

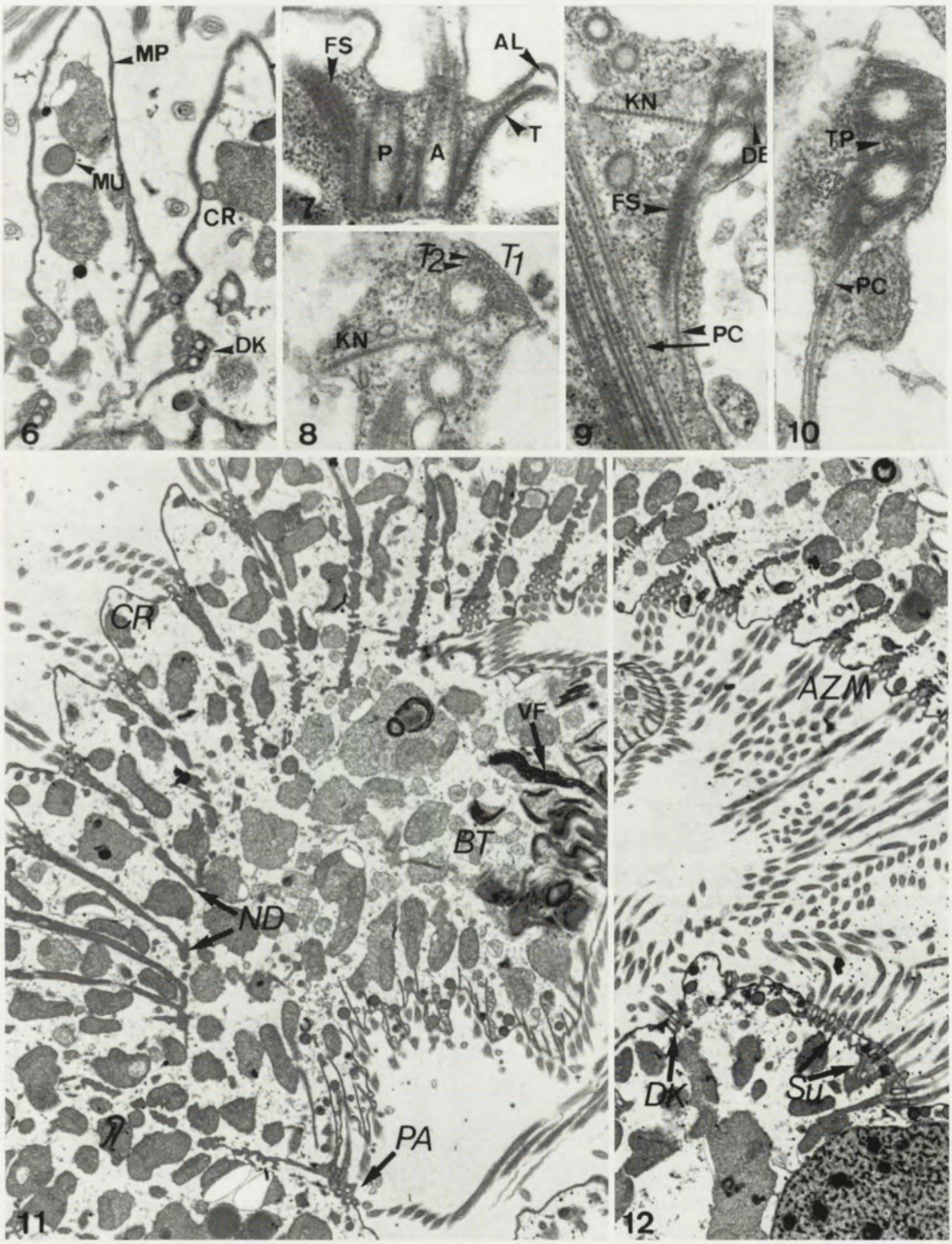
Endoplasme. Il est très vacuolé (Figs. 6, 11, 12) avec des tractus cytoplasmiques contenant des mitochondries de formes variées (Figs. 6, 11, 12) et des proies (Ciliés, Diatomés, Fig. 31).

DISCUSSION

Position systèmatique du Cilié étudié. Le Cilié étudié, à première vue évoque morphologiquement le genre d'eau saumâtre: *Copemetopus*, décrit en 1940 par Villeneuve-Branchon (1940): Cilié de grande taille (300-400 µm) à cinéties nombreuses, et dont le corps déformable, forme à l'avant un lobe ventral au dessus du péristome, l'arrière de la cellule se terminant en pointe mousse. Cependant l'observation de *C. subsalus* par Iftode au microscope électronique (inédit) prouve que notre espèce est bien différente et qu'elle appartient même à un genre différent, plutôt proche de *Condylostoma* par l'absence de lobe ventral surplombant le



Figs. 2-4. Imprégnations argentiques (protargol) de *C. grolieri.* 2 - Vue générale ventrale révélant les cinéties somatiques (Cs) et le système oral avec zone adorale de membranelles (AZM) et organelle paroral (PA) x1700. 3,4 - Grossissement de la figure 2, au niveau buccal avec infundibulum (INF) et némadesme (Fm) issus des cinétosomes de la parorale x3200. 5 - Vue générale ventrale montrant les organelles adoraux (azm), le tapis microtubulaire némadesmal sous-adoral (tm), le macronoyau (ma), des proies dans le cytoplasme (di - diatomée; ci - ciliés) x3200



Figs. 6-10. Electronographies du cortex somatique de *C. grolieri* en coupes tangentielles (Iftode et al. 1982, Ishida et al. 1991, Kennedy 1965, Legrand et Prensier 1976) ou transversales (Iftode et al. 1983). 6 - Deux rangées de dicinétides (DK) implantées entres les crêtes ectoplasmiques (CR) contenant des mucocystes (MU). MP - membrane plasmique x9000. 7 - Paire de cinétosomes d'une dicinétide avec cinétosome antérieur cilié, postérieur non cilié, fibre transverse (T), fibre striée (FS), alvéole (AL) x32800. 8, 9, 10 - Dicinétides avec cinétosomes reliés par des desmoses, rideau de microtubules transverses (T1) doublé de 2 microtubules complémentaires (T2), associé au cinétosome antérieur, 2 microtubules transverses (TP) associés au cinétosome postérieur, comme le rideau de microtubules postciliaires (PC), la fibre retrodesmale striée (FS), la fibre catétodesmale (KN) non striée. x40000, x30800, x40600

Figs. 11-12. Coupes de la région orale de *C. grolieri* montrant les organelles adoraux (AZM) séparés par des crêtes (CR), les némadesmes (ND), issus de cinétosomes de l'AZM et de la parorale (PA), le bourrelet cytoplasmique (BT) séparant AZM et PA, des vésicules pharyngiennes (VF), les organelles antérieurs paroraux (Su), DK - dicinétide somatique. x2300

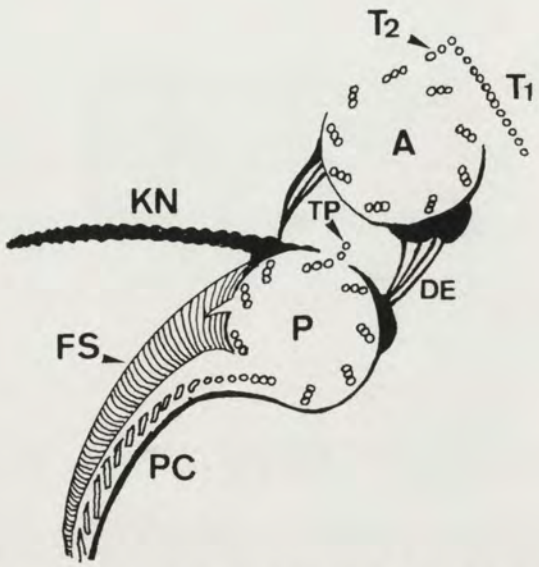


Fig. 13. Schéma d'une dicinétide somatique. Mêmes légendes que dans les figures 6-12

péristome et par la présence d'organelles autonomes apicaux. Elle diffère cependant par l'absence de myonèmes et par la structure de l'organelle paroral, raisons pour lesquelles il ne peut être assimilé au genre *Condylostoma* et à l'espèce *C. tardum* Penard qu'il peut évoquer.

Le genre *Condylostomides* est représenté par des Ciliés d'assez grande taille (250-400 µm) dépourvu de myonèmes, aux cinéties relativement serrées dont quelques postorales, à la cavité péristomienne triangulaire occupant le 1/3 antérieur de la longueur du corps, prolongée en un court infundibulum. La frange adorale d'organelles est semicirculaire peu spiralée à son extrémité postérieure. L'organelle paroral est une double rangée de cinétosomes, antérieurement doublée sur sa droite par de courts segments de cinéties formant des paquets ciliaires distincts.

Condylostomides, comme *Condylostoma* et *Copemetopus* (Iftode et al. 1982), peut être rangé dans la famille des Condylostomidae Kahl in Doflein et Reichenow, 1929.

Cortex somatique. Dans les dicinétides de *C. grolieri*, seul le cinétosome antérieur est cilifère comme chez *Spirostomum ambiguum* (Finley et al. 1964), *Blepharisma undulans* (Kennedy 1965), *Climacostomum virens* (Peck et al. 1975), *Stentor coeruleus* (Huang and Pitelka 1973, Pelvat 1985) et *Eufolliculina* sp. (Mulisch and Hausmann 1984) pour les dicinétides de la région apicale de ce Cilié. Par contre, chez *Condylostoma magnum* (Bohatier 1978, 1979) et *Fabrea salina* (Silva Neto et Grolière 1992) tous les 2 cinétosomes jumelés sont cilifères.

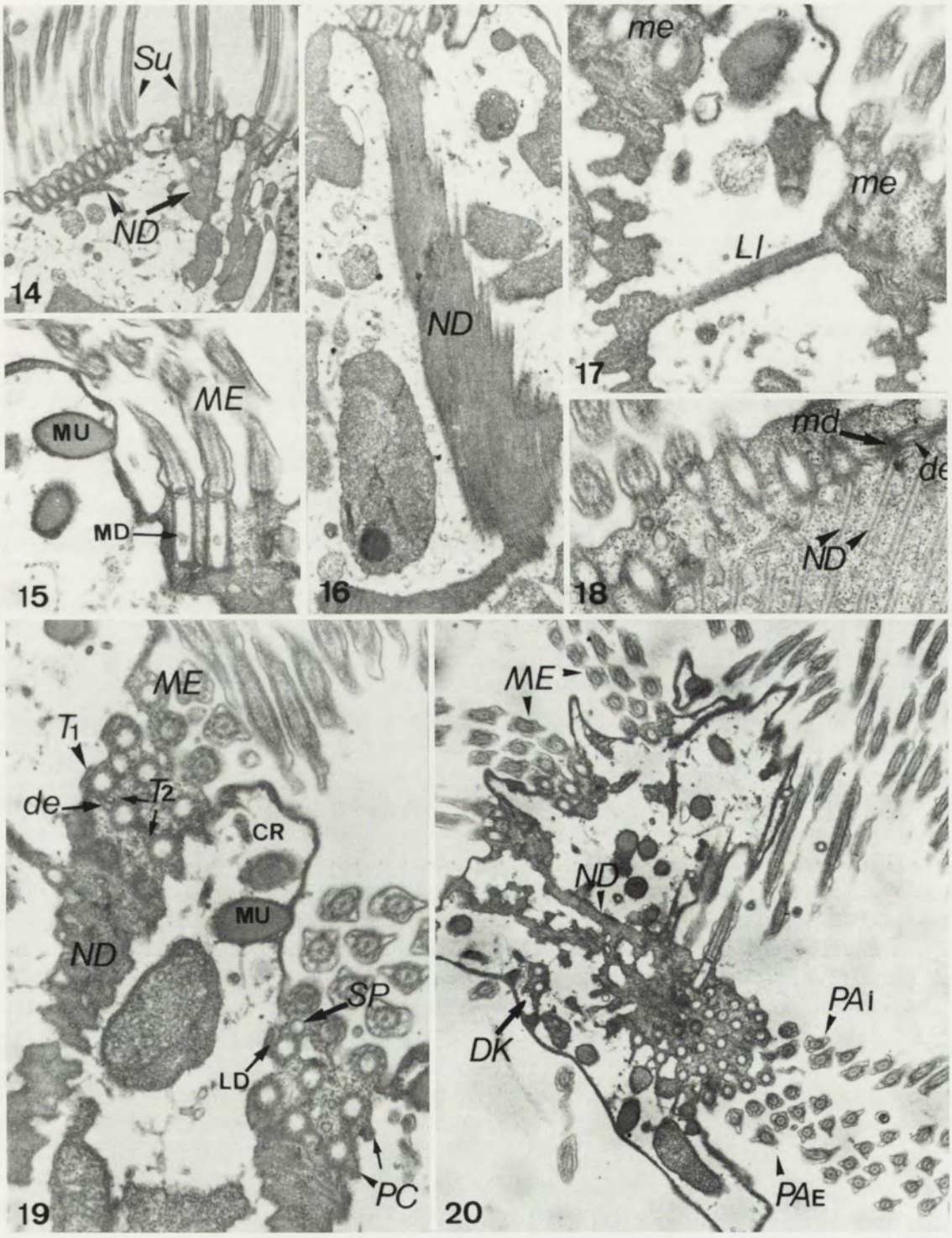
Comme il est typique chez les Hétérotriches (Silva Neto et al. 1992, Silva Neto et Grolière 1992), le cinétosome antérieur est associé à un rideau de fibres transverses (T1) doublé de 2 microtubules complémentaires (T2) et au cinétosome postérieur sont associés 2 microtubules transverses (TP), une fibre cinétodesmale non striée (KN) de Lynn (1981) et un rideau de microtubules postciliaires. Cependant chez *C. grolieri* s'y ajoute une grosse fibre à structure périodique (= fibre striée) qui accompagne à son départ le rideau des fibres postciliaires. Cette particularité est aussi observable chez *Copemetopus* (travail en cours).

Les mucocytes rappellent ceux de *Transitella* (Iftode et al. 1983). Il n'y a pas de myonèmes comme chez d'autres espèces d'Heterotrichida (Bohatier 1978, Finley et al. 1964, Huang and Pitelka 1973, Ishida et al. 1991, Kennedy 1965, Legrand et Prensier 1976, Mulisch et al. 1981, Peck et al. 1975, Silva Neto et Grolière 1992).

Cortex buccal. La frange adorale d'organelles est formée de paramembranelles (Puytorac et Grain 1976) ayant les sytèmes fibrillaires tangentiels associés, comme chez la plupart des Heterotriches: microtubules transverses (T1 et T2) sur les cinétosomes de la rangée gauche de l'organelle, microtubules postciliaires sur ceux de la rangée droite. Comme chez *Eufollicculina uhligi* (Mulisch and Hausmann 1984) et *Fabrea salina* (Mulisch et al. 1981) une lame de matériau dense accompagne les fibres transverses du côté interne.

Les liaisons inter organelles adoraux de *C. grolieri* sont formées par les némadesmes issus des régions proximales des cinétosomes de la rangée gauche des organelles, alors que chez *Condylostoma magnum* ces némadesmes sont issus dans la partie moyenne des cinétosomes (Bohatier 1978). Chez *Phacodinium metchnicoffi* (Silva Neto 1993), *Transitella corbifera* et *Transitella bivacuolata* (Iftode et al. 1983), *Copemetopus* (sous presse), les Armophorida (Mildem Rodrigues de Santa Rosa 1976, Silva Neto et al. 1994) les Clevelandellida (Paulin 1967) et les Odontostomatida (Schrenk and Bardele 1991), les liaisons interorganelles sont assurées par des réseaux microfibrillaires. La particularité représentée par la présence de némadesmes sur les cinétosomes de la rangée gauche ne se retrouve que chez *Transitella* (Iftode et al. 1983) et *Nyctotherus* (Paulin 1967).

L'organelle paroral est formé de 2 rangées de cinétosomes avec, dans le tiers antérieur de l'organelle



Figs. 14-20. Electronographies de la région buccale de *C. grolieri* avec les organelles (ME) associés à des némadesmes (ND). x6900. 15 - Coupe transversale au niveau d'un organelle adoral (ME) de 3 rangées de cinétosomes contenant un matériau dense (MD). Mucocyste - MV x16700. 16,17,18 - Coupes tangentielles d'organelles (ME) montrant les némadesmes (ND) issus de la base (md) des cinétosomes et s'enfonçant en lames profondes dans l'endoplasme, les liaisons intermembranellaires (LI), les desmoses (de) entre cinétosomes au niveau de leur extrémité proximale. x9000; x22400; x21700. 19 - Vue de 2 organelles adoraux (ME) séparés par une crête ectoplasmique (CR) avec mucocyste (MU) montrant les fibres transverses (T1 et T2) de la rangée gauche, une lame dense (LD) entre les rideaux de microtubules T1, les fibres postciliaires (PC) de la rangée droite des organelles, les liaisons intercinétosomiennes (de), les némadesmes (ND), des invaginations membranaires (SP) x19300. 20 - Section de la région orale montrant les organelles adoraux (ME), l'organelle paroral dans le trajet antérieur avec les 2 rangées de cinétosomes internes (PAi) et les rangées de cinétosomes externes (PAe). Des némadesmes (ND) relient la parorale aux adorales. DK : dicinétide somatique. x8600

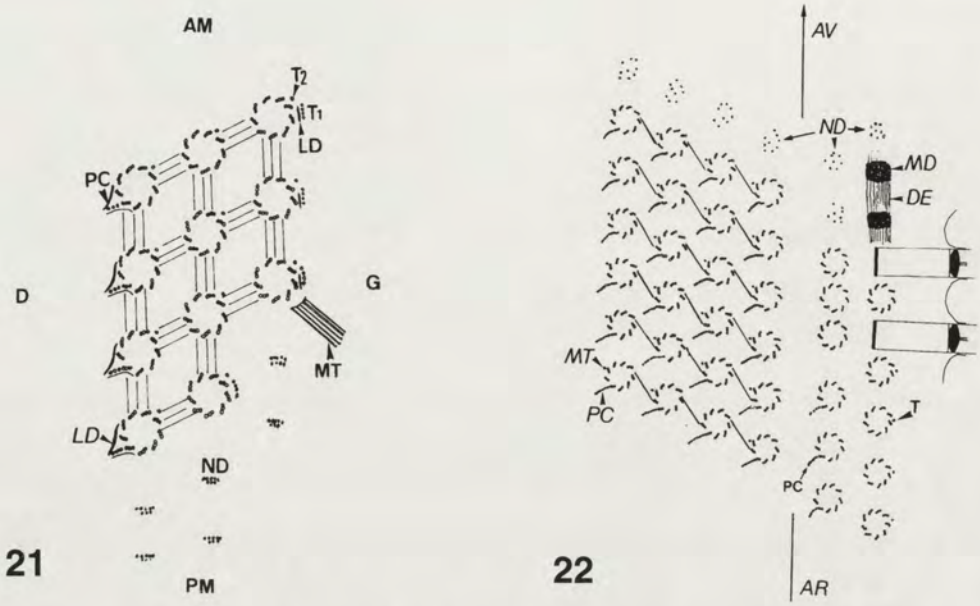


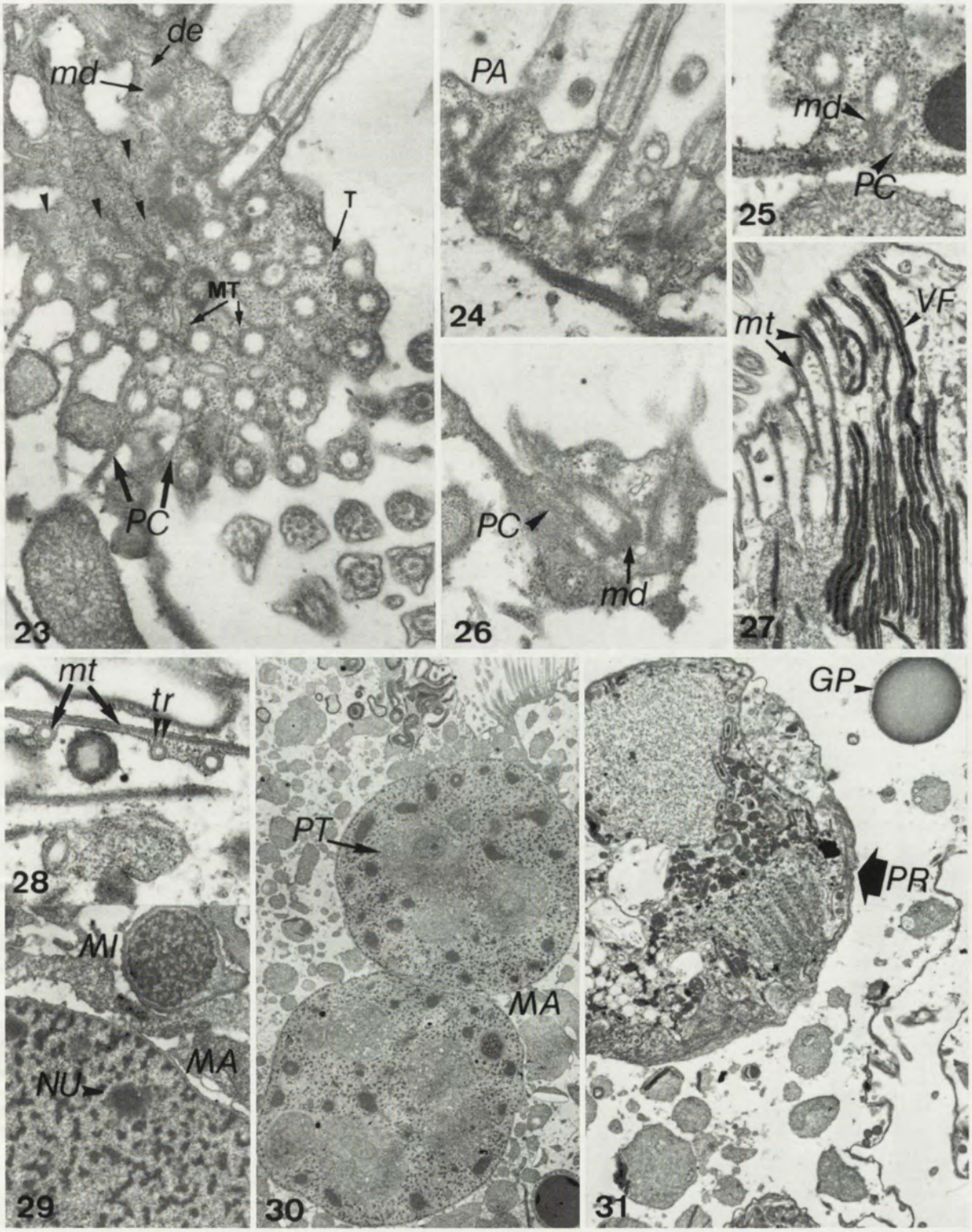
Fig. 21. Schéma de la structure d'un organelle adoral. Mêmes légendes que dans les Figs. 19-20. AM, PM : partie antérieure, postérieure de l'organelle, D, G : droite gauche de l'organelle

Fig. 22. Schéma de la structure de l'organelle paroral dans son trajet antérieur. Mçme légende que dans Figs. 20, 23. AV, AR - avant, arrière de l'organelle

et du côté externe, addition d'une série de courts segments de cinéties (de 4 cinétosomes). Ces paquets infraciliaires bordant la partie antéro-droite du péristome, évoquent les plages cilifères de *Condylostoma magnum* (Bohatier 1978, 1979) dont elles diffèrent par la présence de fibres némadesmales. Chez *Condylostoma,* ce secteur cortical est formé à partir du champ paroral. Il en est peut-être de même chez *Condylostomides* où la stomatogénèse débute par un champ anarchique localisé entre quelques cinéties ventrales, comme chez *Condylostoma.* Cette organisation évoque celle de la parorale de *Condylostoma magnum* (Bohatier 1978), *Phacodinium* (Silva Neto 1993, Silva Neto et al. 1993c), *Fabrea salina* (Silva Neto et Grolière 1992) et *Copemetopus* (travail en cours). Mais chez *Condylostoma magnum,* les courtes cinéties sont insérées dans des sillons et localisées du côté interne de la rangée principale de cinétosomes, et, chez *Fabrea* comme *Phacodinium,* les segments de cinéties sont de 3 cinétosomes le plus souvent, de 1 à 4 chez *Copemetopus.* Tous les cinétosomes de ces cinéties courtes parorales de *C. grolieri* sont associés à un rideau de microtubules postciliaires, comme chez *Fabrea,* et à un microtubule isolé proche du triplet 7. Chez *Phacodinium* et *Condylostoma*, seuls les cinétosomes de la rangée interne ont des fibres postciliaires. Chez *Phacodinium* les cinétosomes de la rangée interne sont orientés perpendiculairement aux cinétosomes des courtes cinéties. Chez *Condylostomides* seulement dans des paires antérieures de la rangée interne des cinétosomes ont ce type de disposition.

Des némadesmes sont issus de la zone proximale des cinétosomes de la parorale de *Condylostomides* comme chez *Fabrea.* Chez *Condylostoma,* il n'y a pas de némadesmes au niveau des cinétosomes de la rangée longitudinale la plus externe. Alors que des microtubules sont associés aux cinétosomes paroraux de *Stentor coeruleus* (Pelvat 1985) et *Eufolliculina uhligi* (Mulisch and Hausmann 1984), chez *Phacodinium* (Silva Neto 1993), *Copemetopus, Parametopus* (Silva Neto et al. 1992, 1993b, 1994) *Caenomorpha* (Mildem Rodrigues de Santa Rosa 1976) et *Sicuophora* (Puytorac et Grain 1968) ce sont des réseaux microfibrillaires à noeuds qui sont présents. Chez *Transitella* (Iftode et al. 1983) et *Nyctotherus* (Paulin 1967) il y a combinaison de ces 2 types de dérivés.

Endoplasme. Appareil nucléaire. Les proies (Ciliés-Diatomées) présentes dans les vacuoles digestives du Cilié permettent de préciser la nature de l'alimentation de ce dernier. Le macronoyau est d'un type assez commun chez les Heterotriches mais par contre la présence fréquente de parasites nucléés ameboides dans le caryoplasme est une originalité.



Figs. 23-31. Electronographies de la région buccale et de l'appareil nucléaire de *C. grolieri.* 23 - Grossissement de la Fig. 20 pour révèler les microtubules postciliaires (PC), les microtubules transverses (MT), les némadesmes (flèches), materiel dense (md), les desmoses (de). x22600. 24, 25, 26 - Sections au niveau de la parorale (PA) interne avec des cinétosomes implantés perpendiculairement l'un par rapport à l'autre (Silva Neto et al. 1993a, 1993b), fibres postciliaires (PC) accompagnés de matériau dense (md). x21 500, x37600, x30300. 27 - Vacuoles pharyngiennes (VF) avec microtubules (mt). x12600. 28 - Microtubules (mt) associés à 2 microtubules (tr) de faible diamètre. x22600. 29-30 - Appareil nucléaire avec macronoyau (MA) contenant des nucléoles (NU) et des parasites nuclées (PT) et micronoyau (MI) x9400, x2300. 31 - Proie (PA) envacuolée dans l'endoplasme et inclusion (pigment ?) (GP). x3600

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Effects of Cadmium on *Uronema marinum* (Ciliophora, Scuticociliatida) from Antarctica

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Summary. The toxic effects of cadmium, a heavy metal cation which is important as an environmental pollutant, were studied on cultures of *Uronema marinum*, a scuticociliatid isolated from seawater and sandy sediments collected in Terra Nova Bay, Antarctica. *Uronema marinum* cells were sensitive to cadmium to a dose as low as 2µg/ml (18 µM). The metal is mostly accumulated in the sediment fraction, probably linked to membranes and cytoplasmic organelles. Transmission electron microscopy indicated generalized damage of cell ultrastructure. The lack of specific Cd-binding compounds may explain the toxic effect observed.

Key words. *Uronema marinum*, Antarctica, cadmium, accumulation, ultrastructure.

INTRODUCTION

Cadmium (Cd) is an ubiquitous element found in close association with zinc in the earth's crust. Mining and industrial activities have probably increased its abundance in the biosphere during the last few decades. Thus, there is general concern that environmental exposure to cadmium has been continuously increasing. Oceanic concentrations of Cd are of the order of 0.02-0.12 µg/l (Bruland et al. 1979), while in estuarine waters dissolved concentrations exceeding 50 µg/l have been measured (Bryan and Langston 1992). The principal dissolved forms of Cd are chloride complexes, but the most easily available species is probably the free Cd ion, the proportion of which increases with decreasing salinity (Cross and Sunda 1985). Dissolved Cd is sometimes remobilized from sediments and is a very important source of pollution. Thus, marine organisms are continually exposed to variable concentrations of Cd in water. It is well-known that, although Cd only occurs in the marine environment in trace concentrations, most marine organisms accumulate it rapidly in non-toxic forms (Bryan 1976, Viarengo and Nott 1993). For example, data on Antarctic krill and the bivalve *Adamussium colbecki* indicate relatively high contents of Cd, despite its quite low concentration in Antarctic seawater (Cognetti Alfinito et al. 1991). In fact, total dissolved Cd concentrations in the Antarctic seawater of the Ross Sea ranged between 0.009 and 0.078 µg/l; higher values, up to 0.12 µg/l, have been recorded near the coast and the Italian station, which may reflect anthropogenic contamination (Saini et al. 1989, Capodaglio et al. 1991).

It thus appears of great interest to study the degree of tolerance towards contamination by Cd in Protozoa,

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an important component of the aquatic food chain, in remote areas far from important sources of contamination, such as the Antarctic environment.

In the present study the scuticociliatid *Uronema marinum* Dujardin, 1841, isolated from material collected in the Ross Sea (Antarctica) (Coppellotti 1990), was exposed to cadmium in laboratory conditions. The degree of tolerance to cadmium with respect to growth, metal accumulation and ultrastructural organization was examined in this organism, which lives in a harsh environment characterized by extremes of temperature and partial pressure of oxygen. Data are compared with those recorded in other Protozoa.

MATERIALS AND METHODS

Organism and culture methods

Samples of seawater and sandy sediments from which *Uronema marinum* Dujardin, 1841, was isolated were collected from a small cove near the Italian Antarctic base at Terra Nova Bay (Ross Sea, 74°42'S, 164°06'E) at the beginning of 1988. At the time of collection, the following environmental parameters were recorded: salinity, 35‰; temperature,-1.8°C; pH 8.1-8.2.

The cells were cultured in the laboratory at 2°C, in natural seawater (salinity 35‰), filtered with 0.22 µm unit Sterivex-GS (Millipore), enriched with 0.005% proteose-peptone, which permits limited growth of unidentified bacteria, as previously reported (Coppellotti 1990).

Cadmium was added to seawater as $CdCl_2 \cdot H_2O$ at final concentrations of 2, 4, 8, 10, 15 and 20 µg Cd/ml, corresponding to 18, 35, 70, 90, 130 and 180 µM. Cells from an initial log phase were inoculated in fresh culture medium up to 25 ml in small cups for growth curves, and up to 500 ml in flasks for biochemical investigations, to give an initial density in both cases of 600 cells/ml.

Growth was monitored daily to day 15 by counting cells in a hemocytometer (Bürker chamber). Both ultrastructural and biochemical studies were performed at day 7.

Metal and chromatographic analyses

Uronema marinum cells were pelletted by centrifugation at 3,000 g for 40 min at 2°C and washed repeatedly in cold seawater to separate cells from bacteria.

Cells were homogenized in the cold with a Polytron homogenizer in 50 mM Tris-HCl buffer, pH 7.5. The homogenate was centrifuged at 48,000 g for 2h at 2°C and the sediment was washed with the same buffer.

Metal contents (Cd and Zn) in the supernatant were measured by atomic absorption spectroscopy with a Perkin-Elmer mod. 4000 spectrophotometer, without further processing. Metal contents in homogenates and sediments were measured after wet ashing in concentrated HNO_3 (AristaR grade 60%) in a Teflon bomb for 2 h at 160°C. Digested material was diluted up to 5 ml with MilliQ water. All measurements were corrected for background absorption using reagent blanks. Every datum is the mean of three experiments.

250 µl aliquots of supernatant were processed on a Beckman High Performance Liquid Chromatography (HPLC) mod. 341 equipped with a Waters Protein Pak 60 column (Millipore) with the aim of separating metal-linking fractions. The elution buffer was 10 mM Tris-HCl containing 0.2 M NaCl, pH 7.5, at a flow rate of 0.5 ml/min.

Light microscope observations

Ciliates from control and cadmium-exposed cultures were withdrawn with a pipette and fixed with glutaraldehyde 5% (v/v) in seawater.

Light microscope observations were performed with a Leitz Diaplan microscope equipped with a fluorescence apparatus. The nuclear apparatus was observed after staining with 4'-6-diamidino-2-phenylindole· 2HCl (DAPI), as previously reported (Coppellotti 1990).

Transmission electron microscope observations

Ciliates were collected by centrifugation at 1,000 g for 5 min at 2°C from control and cadmium-exposed cultures (4 µg and 10µg/ml, i.e., 35 and 90 µM Cd) 7 days after inoculation. Cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 with 6% (w/v) sucrose at 0°C for 45 min. They were then washed several times with the same buffer and post-fixed in 1% (w/v) OsO_4 for 1 h. The procedure followed was according to Dallai et al. (1985).

The material was dehydrated in a graded series of ethanol, soaked in propylene oxide, and embedded in Epon. The thin sections, cut with an LKB Ultratome, were stained with uranyl acetate for 15 min and lead citrate for 5 min (Reynolds 1963), and examined under a Hitachi H600 electron microscope.

RESULTS

Growth curves and metal uptake

Results on the effects of Cd on cell growth are shown in Fig. 1. Addition of Cd affected the growth rate even at the lowest dose used (2 µg/ml); inhibition depended on Cd concentration. As Fig.1 shows, a decrease in the number of cells was recorded in the first 2-3 days of culture and subsequently, after 3-4 days, partial recovery was evident. The lag phase, in which cells were largest, was extended. These effects were marked at the highest concentration used.

Reduplication time between days 4 and 5 was about 12 h in controls, and was enhanced to 36 h and 55 h in the presence of 2 µg and 4 µg Cd/ml, respectively.

Our light microscope observations on *Uronema* cultures revealed that the phenomenon of conjugation, previously described by Coppellotti (1990), which appears between days 5 and 7 in controls, was delayed starting at day 8 in cells exposed to 2 µg Cd/ml. At higher doses, conjugation was abolished, at least over a growth period of 15 days. DAPI fluorescent images (Figs. 2-8) clearly indicate alterations of the nuclear apparatus: absence of micronucleus, shift of the micronucleus from its original

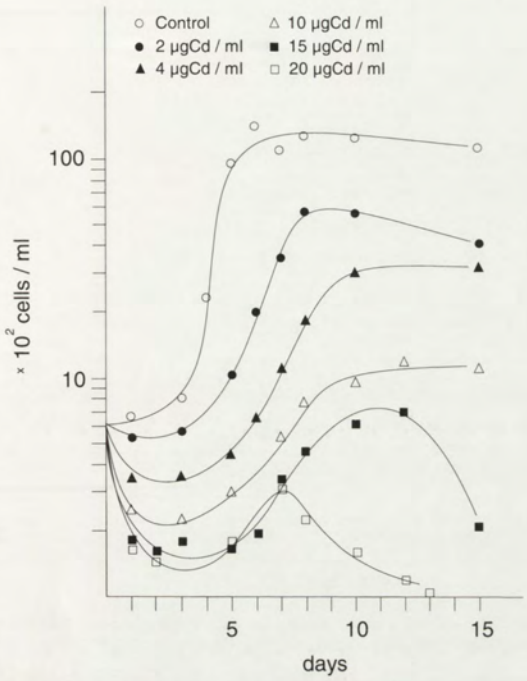


Table 1

Metal contents (µg/g dry wt) in control *Uronema marinum* cells and cells exposed to Cd (2, 4, 8 µg/ml) at day 7

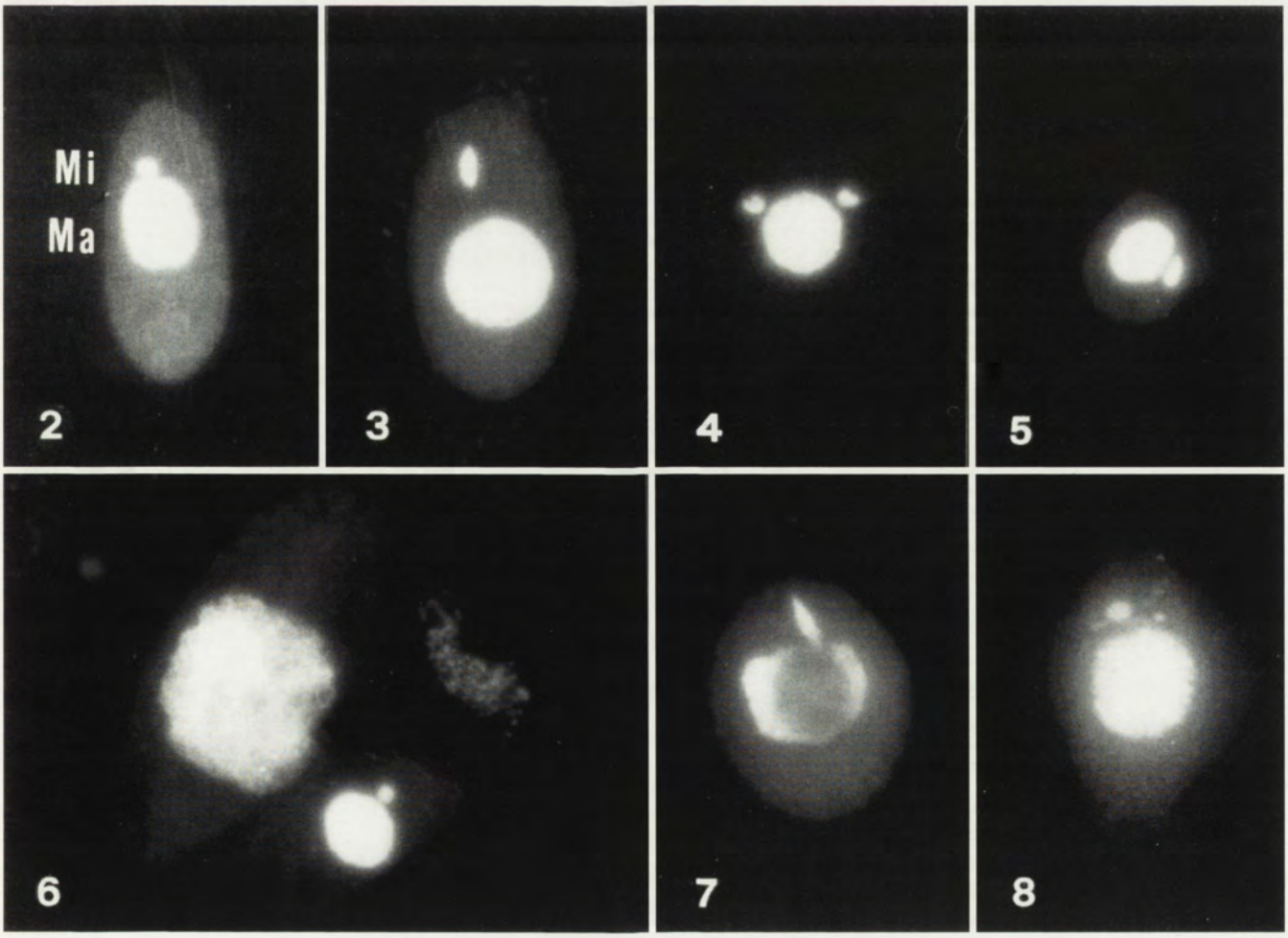
Treatment	Cd	Zn
Controls	0	115
2 µg Cd/ml	380	100
4 µg Cd/ml	410	95
8 µg Cd/ml	465	40

position, two micronuclei, and disaggregation of nuclear material.

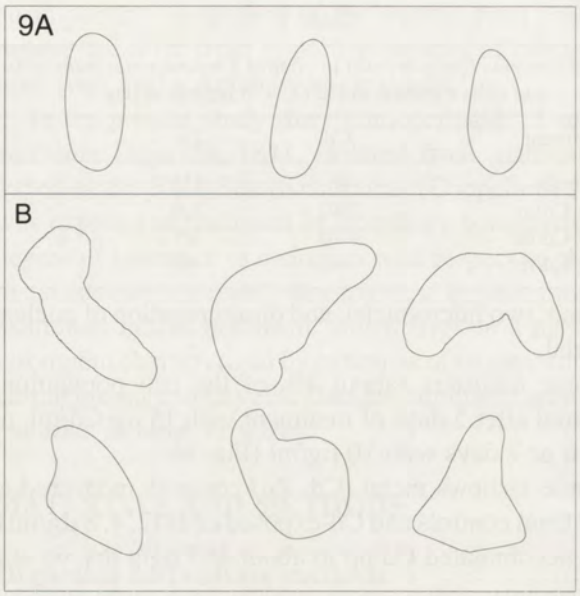
Some monsters (about 1% of the cell population) appeared after 5 days of treatment with 15 µg Cd/ml, or after 6 or 7 days with 10 µg/ml (Fig. 9).

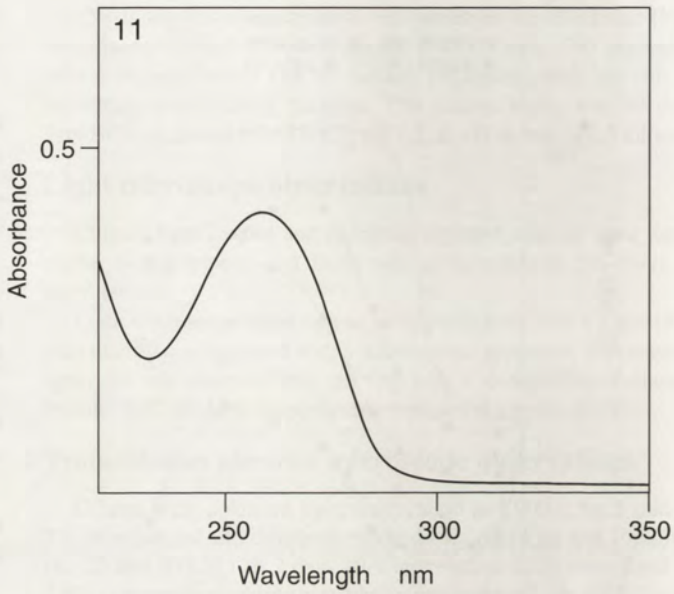
Table 1 shows metal (Cd, Zn) contents recovered at day 7 from controls and Cd-exposed cells (2, 4, 8 µg/ml). Cells accumulated Cd up to about 465 µg/g dry wt at a

Fig. 1. Concentration-dependent effects of cadmium chloride on cell proliferation of *Uronema marinum* up to day 15 of culture



Figs. 2-8. DAPI fluorescent images of nuclear apparatus at day 7 (x 1,000). Macronucleus (Ma); micronucleus (Mi). 2 - control cell; 3 - Cell exposed to 2µg Cd/ml; 4-6 - cells exposed to 15µg Cd/ml; 7-8 - cells exposed to 20µg Cd/ml





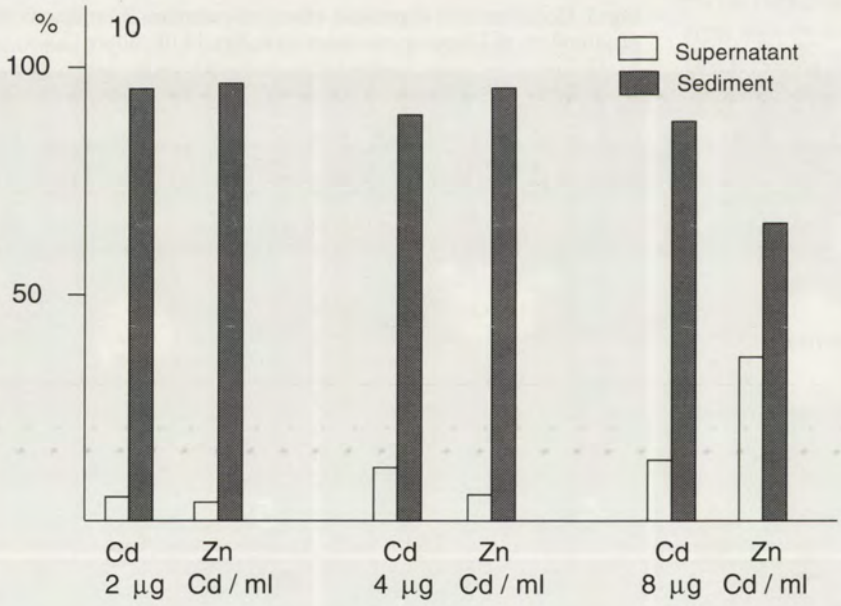


Fig. 9. Cell profiles of control cells (A) and monsters (B) induced by exposure to doses of at least 10µg Cd/ml. Bizarre shapes indicate repeated abolition of cell division. Drawings from light micrographs of cells fixed with glutaraldehyde (x 500)

Fig. 10. Percentage distribution of metals (Cd and Zn) between soluble and particulate fractions after homogenization of cells at day 7

Fig. 11. Ultraviolet spectrum of a Cd-linking fraction isolated by HPLC chromatography

dose of 8 µg Cd/ml: this value was only 20% higher than that recorded at a dose of 2 µg Cd/ml. Treated cells showed Zn depletion.

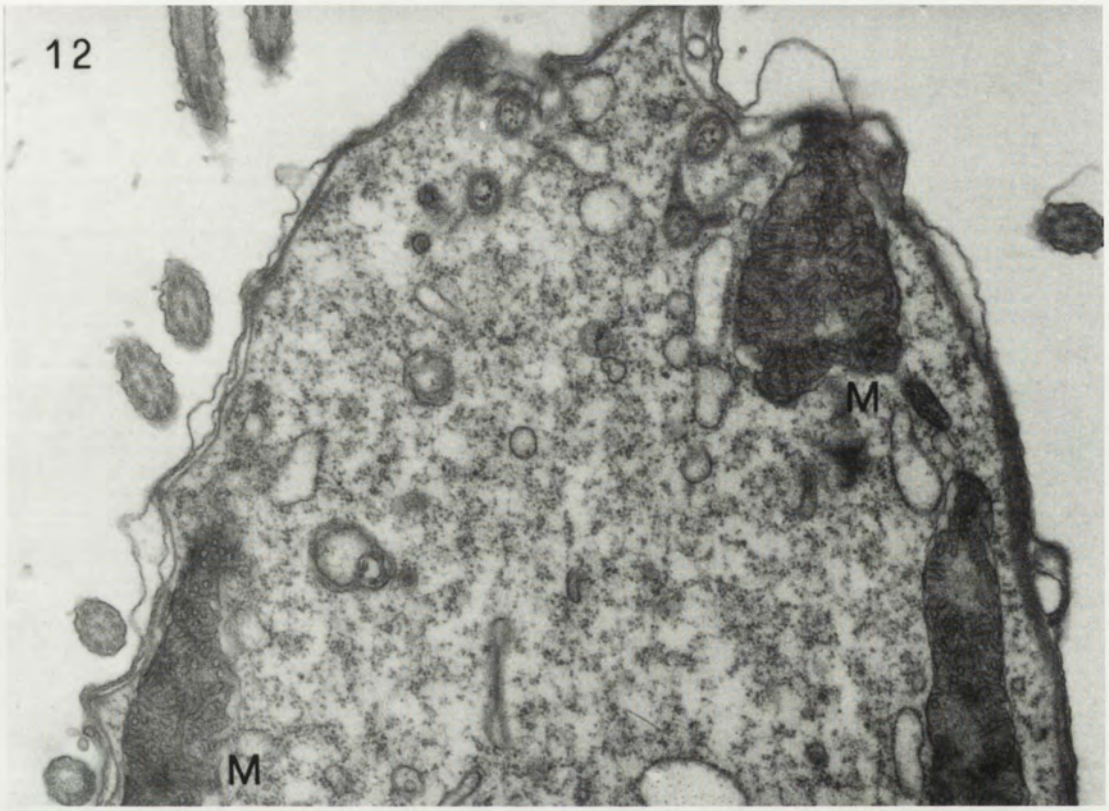
The percentage distribution of both metals between soluble and particulate fractions is shown in Fig.10: the highest amounts were always recovered from the particulate fraction.

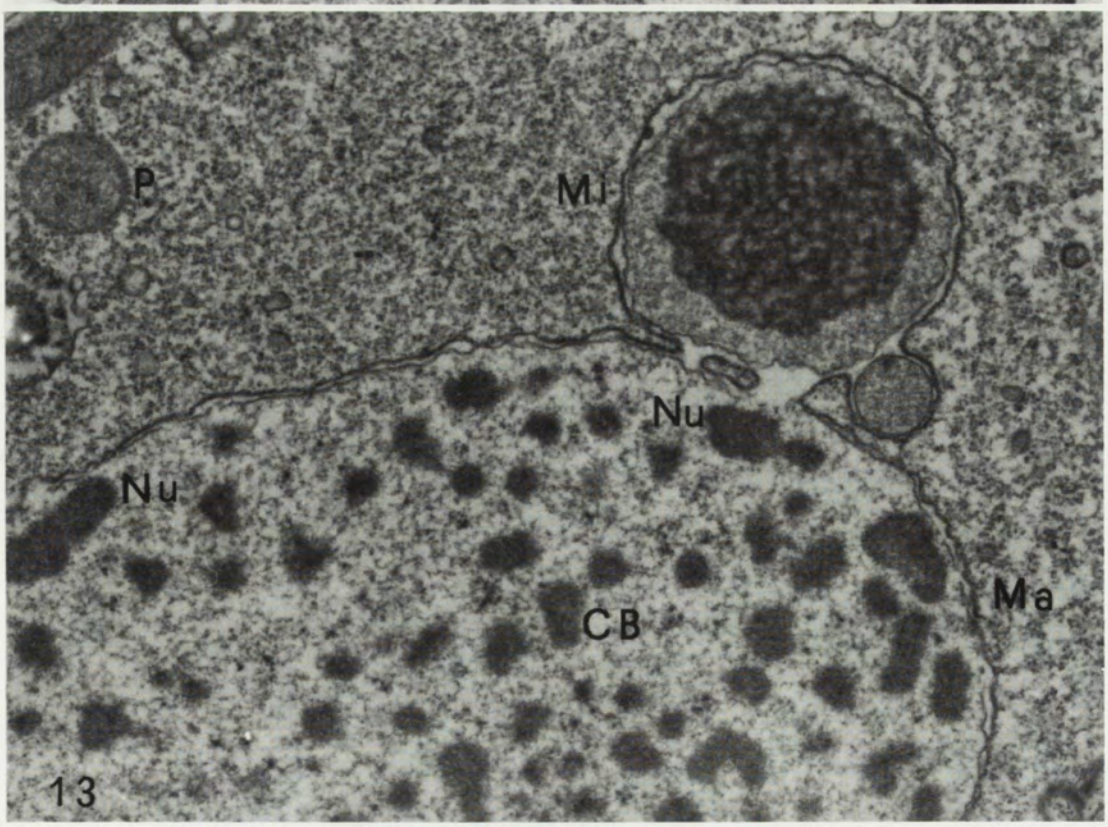
No specific Cd-binding ligands were isolated from supernatants, because Cd was broadly distributed in the various fractions. The ultraviolet absorption spectra of the Cd-linking fractions showed high absorbance at 260 nm, indicating the possible presence of nucleic acids (Fig.11).

Transmission electron microscope observations

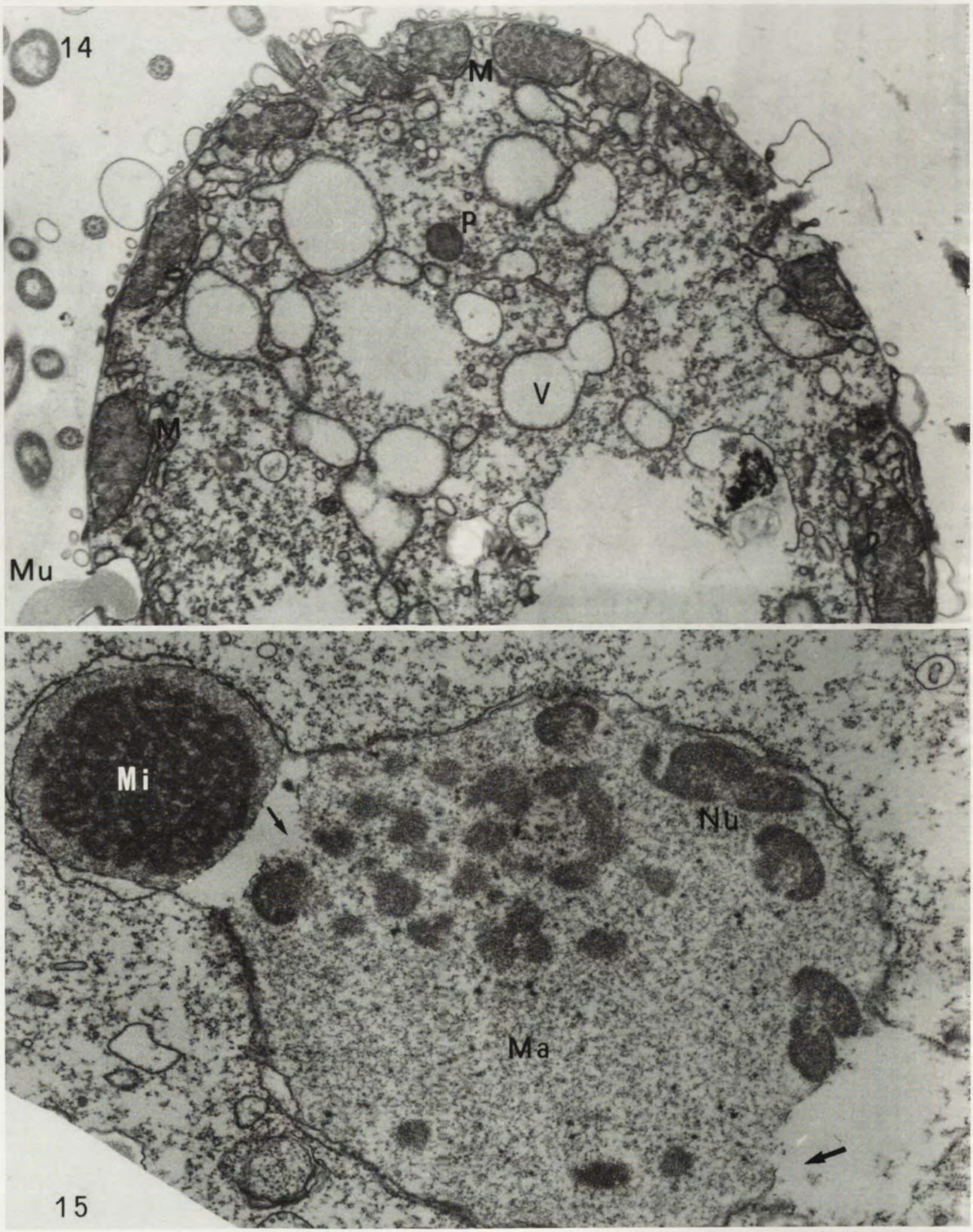
Ultrastructural study of *Uronema marinum* cells at day 7 of exposure to two different doses of Cd (4 and 10 µg Cd/ml) revealed some changes in the cytoplasm and nucleus, especially at the higher dose, in comparison with control cells (Figs. 12, 13). Extensive vacuolization of the cytoplasm, and highly electron-dense granules provided with membranes were detected (Figs. 14, 16, 17).

Disintegration of cristae may be observed in mitochondria (Figs. 16, 17).

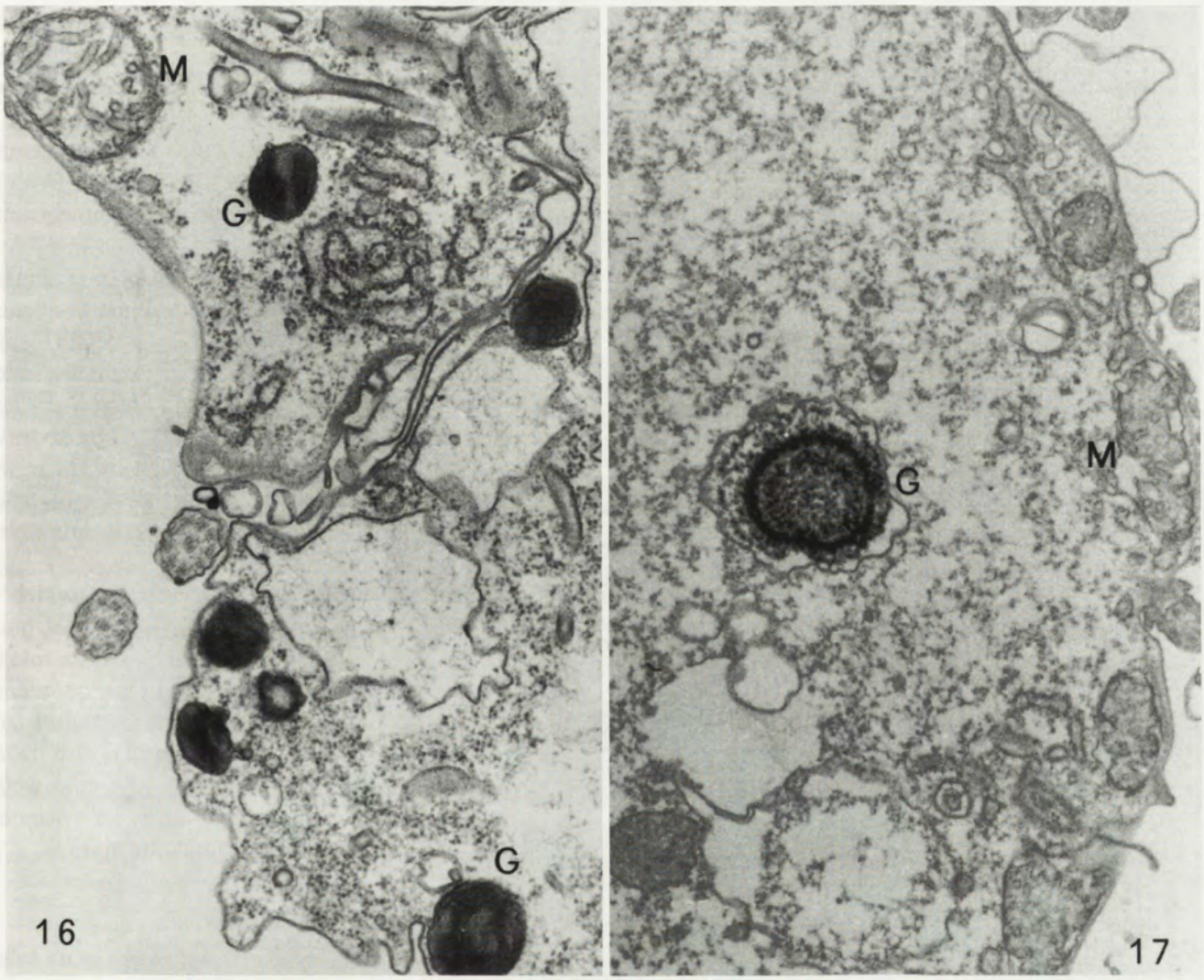




Figs. 12-13. Ultrastructural morphology of *Uronema marinum* control cell (x 20,000). 12 - Mitochondria (M) are cortically oriented. Note absence of mitochondria deeper in cytoplasm. 13 - Nuclear apparatus. Chromatin in micronucleus (Mi) is condensed. Macronucleus (Ma) contains large peripheral nucleoli (Nu) with typical granular appearance, chromatin bodies (CB), and small interchromatin granules. Peroxisome (P)



Figs. 14-15. Electron micrographs of cells exposed to 10µg Cd/ml at day 7 (x 20,000). 14 - Cross section. Note intermeridional distribution of mitochondria (M) with loss of cristae, high vacuolization of cytoplasm (V), extruded mucocyst (Mu), and peroxisome (P). 15 - Macronucleus (Ma) containing irregular nucleoli (Nu), fused into large bodies. Macronucleus membranes are partially disintegrated (arrows)



Figs. 16-17. Electron micrographs of cell exposed to 4µg Cd/ml (Fig. 16; x 27,000) and 10µg Cd/ml (Fig. 17; x 24,000). Note mitochondria (M) with loss of cristae and granules (G) provided with membranes

As for the nuclear apparatus, *Uronema* cells are endowed with a macronucleus and a micronucleus, both interconnected (Fig. 13). It can be seen that the micro- and macronuclear outer membranes join at several points and are similar in appearance to those of *Tetrahymena*, according to the description of Kaneshiro and Holz (1976) for some *Uronema* species. Numerous granular nucleoli are visible at the periphery of the macronucleus, and chromatin bodies and interchromatin granules are scattered throughout the nucleoplasm.

In the macronucleus of treated cells, some changes in the fine nucleolar organization were observed with respect to controls: the nucleoli aggregate and have irregular shapes, and the chromatin granules are reduced in number (Fig.15).

Disaggregation of the nuclear envelope is also evident in Cd-exposed cells (Fig.15).

DISCUSSION

Uronema marinum cells turn out to be very sensitive to the toxic effects of cadmium. The growth of *Uronema* is slowed from a dose of 2 µg Cd/ml (18 µM Cd), which induces a generation time three times longer than in controls. Such treatment stimulates Cd accumulation up to about 465 µg/g dry wt at day 7 of culture at the highest dose tested (8µg Cd/ml), while Zn is depleted. It is widely assumed that Cd treatment may disturb the normal metabolism of an essential metal, like Zn, and cause symptoms of Zn deficiency as well as accumulation

(Albergoni et al. 1980, Chmielnicka and Cherian 1986). The observed depletion is probably related to competition by Cd, which substitutes Zn in some Zn-containing proteins, later releasing it in a biologically inactive form (Pool 1981).

The degree of intracellular accumulation of Cd is not very high, when compared with values recorded for other ciliates such as *Tetrahymena pyriformis* and *Tetrahymena thermophila* (Piccinni et al. 1987, Piccinni et al. 1990) or *Oxytricha granulifera* (Piccinni et al. 1992), and for the flagellate *Euglena gracilis* (Coppellotti 1989). High Cd accumulation in all these organisms is related to specific Cd-linking compounds such as metallothioneins or glutathione, which are involved in reducing the intracellular presence of the metal in the toxic ionic form and which are responsible for the high tolerance shown by cell cultures. Cd is mainly compartmentalized in the particulate fraction in *Uronema* cells, probably in granules or membranous structures. Compartmentalization of metals in cytoplasmic granules or membrane-bound vesicles is in fact widespread in organisms and is considered to be one of the tolerance mechanisms against metal toxicity (Piccinni 1989, Viarengo and Nott 1993). As regards Ciliophora, toxicity has been extensively studied in *Tetrahymena* (Nilsson 1989 and references therein).

Our attempts to isolate specific Cd-binding ligands from the cytosoluble fraction in *Uronema* gave unsatisfactory results. However, the lack of such compounds may explain the toxic effects of Cd on cell ultrastructure.

Ultrastructural study of *Uronema* cells exposed to 4 and 10 μg Cd/ml for 7 days revealed changes in the cytoplasm (extensive vacuolization, highly electron-dense granules provided with membranes), in the mitochondria (loss of cristae) and in the macronucleus (aggregation of nucleoli, disaggregation of the nuclear envelope) similar to those described for *Tetrahymena* (Pyne et al. 1983, Krawczyńska et al. 1989). Observed changes in the macronucleus suggest inhibition of ribosomal RNA synthesis, responsible for a decreased growth rate. Alterations in the nuclear apparatus, such as loss of the micronucleus or presence of two micronuclei, also observed in light microscopy with fluorescence, are probably responsible for the lack of conjugation in cultures exposed to the highest Cd concentrations. Disturbed conjugation shows the importance of toxicity for the genetic renewal of a cell population. It has been also recorded in *Euplotes harpae* from the Baltic after exposure to high levels of heavy metals such as lead or zinc (Boikova 1990).

The unimicronucleate condition is a remarkably stable feature in ciliates endowed with only one micronucleus in their nuclear apparatus. Increased numbers of micronuclei are generally considered to be a consequence of reorganization, which regularly occurs in starving ciliates as *Euplotes* (Luporini and Bracchi 1973, Fleury and Fryd-Versavel 1981). This process and the development of cells characterized by bizarre forms may be considered not as specific to cadmium treatment but as a stereotyped morphogenetic response to aggression such as heat or exposure to a heavy metal (Fleury et al. 1983, Nilsson 1992). In this case, it indicates interference by Cd with proper assembly of new cortical structures, perhaps due to disorder induced by abortive cell divisions. The perturbation elicited by Cd in the organization of cytoskeletal elements like microtubules and microfilaments has also been reported in cultures of Swiss 3T3 cells (Li et al. 1993).

The temperature used in the experiments, which is near the natural temperature of Antarctic seawater, must be noted. This low temperature probably plays a role in slowing cell metabolism, as suggested by the quite long lag phase and reduplication time, even in control cultures. Intracellular cadmium accumulation is also maintained at quite low levels. Adaptation to a cold water environment suggests the existence of peculiar physiological features in this cosmopolite species.

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Effect of G-protein Activating Fluorides (NaF, AlF_4 and BeF_3) on the Phospholipid Turnover and the PI System of *Tetrahymena*

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Summary. G-protein activating fluorides (NaF, AlF_4 and BeF_3) influence the synthesis, metabolism and breakdown of phospholipids in *Tetrahymena pyriformis*. $AlCl_3$ and $BeCl_2$ also influence the parameters mentioned above, however in a different manner. The experiments suggest the presence of G-protein in *Tetrahymena* possible and point to the function of a G-protein regulated PI system at this low level of phylogeny.

Key words. Phosphatidyl inositol, *Tetrahymena*, G-protein, fluorides, second messenger.

INTRODUCTION

The ciliated protozoan *Tetrahymena pyriformis* possesses the phosphatidyl inositol (PI) system in addition to other second messenger systems (Csaba 1980, Kovács and Csaba 1994, Kovács et al. 1989). Incorporation of labelled phosphorus into phospholipids (as an entry into members of PI system) was detected following treatments with ^{32}P-Na orthophosphate. Likewise, treatments with phorbol ester which also changed the ^{32}P incorporation. The above mentioned facts and the presence of the functioning adenyl cyclase-cyclic AMP system (Csaba 1985, Csaba et al. 1987) suggest that *Tetrahymena* also possess transducer G-proteins between the membrane receptor and the executive enzyme like other unicellular organisms (Cassel et al. 1991, Forney and Rodkey 1992, Lilly et al. 1993) and eukaryote cells at higher phylogenetic levels.

Aluminium fluoride (AlF_4) and beryllium fluoride (BeF_3) complexes, which are formed in the simultaneous presence of $AlCl_3$ or $BeCl_2$ and NaF, are able to activate the heterotrimer G-proteins as they can link both to the nucleotide binding site of G alpha subunit and to the GDP. These complexes have the capacity to express similar effects on phospholipase C like the gamma phosphate of GTP (Antonny and Chabre 1992, Marc et al. 1988, Chabre 1990). In this way treatments with AlF_4 and BeF_3 complexes could alter the quantities and the ratios of individual members of the PI system.

NaF as a nucleophile reagent is able to inhibit several enzymes, it prevents the autophosphorylation of the insulin receptor and also inhibits the activity of tyrosine kinase (Vinals et al. 1993). In addition, like AlF_4 and BeF_3 complexes NaF has influence on the activity of G proteins (Garcia et al. 1992).

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On the basis of the above mentioned data from the literature we investigated the PI system and the turnover of phospholipids in *Tetrahymena* cells. Our question was weather or not the activation of G-proteins has a role in the machinery of the PI system at a unicellular level. By this indirect way can show the probable presence and role of G-protein.

MATERIALS AND METHODS

Tetrahymena pyriformis GL cells in the logarithmic phase of growth were tested. The cultures were grown in culture medium containing 0.1% yeast extract and 1% Bacto tryptone (Difco, Michigan, USA) at 28°C.

Assay of ^{32}P incorporation

The following substances were added to 25 ml suspension of *Tetrahymena* culture (cell-density 10^6/ml) forming different groups:

a) NaF - 10 mM
b) NaF - 20 mM
c) NaF - 40 mM
d) $AlCl_3$ - 10 µM
e) NaF - 10 mM + $AlCl_3$ - 10 µM
f) NaF - 20 mM + $AlCl_3$ - 10 µM
g) NaF - 40 mM + $AlCl_3$ - 10 µM
h) $BeCl_2$ - 10 µM
i) NaF - 10 mM + $BeCl_2$ - 10 µM
j) NaF - 20 mM + $BeCl_2$ - 10 µM
k) NaF - 40 mM + $BeCl_2$ - 10 µM
l) untreated control group

After 30 minutes 60 µl (12 MBq) ^{32}P-Na-orthophosphate (Izinta, Budapest, 7 GBq/mmol) was added to the 25 ml of *Tetrahymena* suspension. After 5, 10, 20 and 30 minutes 5 ml of 10% trichloroacetic acid was added to 5 ml samples at 4°C. After 20 minutes stay on 4°C the samples were centrifuged and the pellet was extracted with 2 ml of acidic chloroform-methanol solution (chloroform:methanol:HCl = 10:20:0.2) at room temperature. Subsequently the samples were partitioned by addition of 0.6 ml chloroform and 0.6 ml water and the organic (chloroform) phase was dried under nitrogen flow. Then the samples were dissolved in 20 µl chloroform:methanol = 6:1 and the phospholipids were isolated by thin-layer chromatography on oxalate activated silicagel (DC Fertigplatten Kieselgel, Merck, Darmstadt, Germany) with a solvent containing chloroform:acetone:methanol:acetic acid:water = 40:15:13:12:8. When chromatograms became dry they were covered with Kodak TMG X-ray film. The spots of the developed film corresponding to the ^{32}P containing phospholipids were analyzed using a Pharmacia UltraScan XL laser densitometer.

Investigations on the breakdown (transformation) of ^{32}P containing phospholipids

To 35 ml suspension of *Tetrahymena* cultures 100 µl (20 MBq) ^{32}P-Na orthophosphate (7 GBq/mmol) was added (cell density 10^6 cell/ml). One hour later the cells were washed (with centrifugation) three times with fresh culture medium and then the samples were diluted to 35 ml with the fresh culture medium. This time was considered as the 0 point of time. After this one of the following substances was added to an individual culture:

a) NaF - 10 mM
b) NaF - 20 mM
c) NaF - 10 mM + $AlCl_3$ - 10 µM
d) NaF - 10 mM + $BeCl_2$ - 10 µM
e) untreated, control group

Five ml samples were taken at time points 1, 5, 15, 30 and 60 minutes. At 0 time we took a sample from the untreated control group. The samples were extracted, chromatographed and analyzed as it was mentioned in Materials and Methods section 1.

Each group (1 and 2) has been repeated thrice. The data of the Figures represent the mean of the three experiments. Significance were evaluated by Student t test.

Table 1

Effects of the single treatments on the incorporation of ^{32}P into the phospholipids. Values are means of 3 experiments (%of total incorporated ^{32}P)

Control	PIP_2	PIP	PI	PA	PE
5'	0	0	0	0	0
10'	0	0	0	1.75 ±0.13	5.5 ±0.41
20'	1.55 ±0.08	1.1 ±0.08	3.95 ±0.12	3.55 ±0.22	14.4 ±1.3
30'	1.4 ±0.1	1.32 ±0.12	4.97 ±0.23	3.95 ±0.18	15.15 ±1.18
NaF 10 mM					
5'	0	0	0	0	0
10'	1.7 ±0.11	2.5 ±0.11	0	1.3 ±0.09	3.95 ±0.6
20'	2.1 ±0.09	1.0 ±0.07	6.2 ±0.88	3.2 ±0.1	12.5 ±0.77
30'	2.2 ±0.12	1.8 ±0.05	9.9 ±0.79	2.1 ±0.13	18.3 ±1.11
NaF 20 mM					
5'	0	0	0	0	0
10'	0	0	0	0	0
20'	0	1.6 ±0.09	0	0	1.8 ±0.1
30'	1.0 ±0.07	0.5 ±0.05	2.2 ±0.09	0	2.8 ±0.16
$AlCl_3$					
5'	0	0	0	0	0
10'	1.2 ±0.06	0	0	0	4.0 ±0.3
20'	1.3 ±0.1	0.5 ±0.03	1.6 ±0.1	1.5 ±0.09	8.3 ±0.41
30'	1.3 ±0.12	1.7 ±0.09	4.7 ±0.23	3.15 ±0.26	14.9 ±1.6
$BeCl_2$					
5'	0	0	0	0	1.8 ±0.4
10'	0	0	0	0	2.7 ±0.09
20'	1.5 ±0.07	0	5.2 ±0.4	4.7 ±0.3	11.8 ±0.9
30'	2.0 ±0.11	1.8 ±0.07	6.4 ±0.55	5.5 ±0.34	14.0 ±1.35

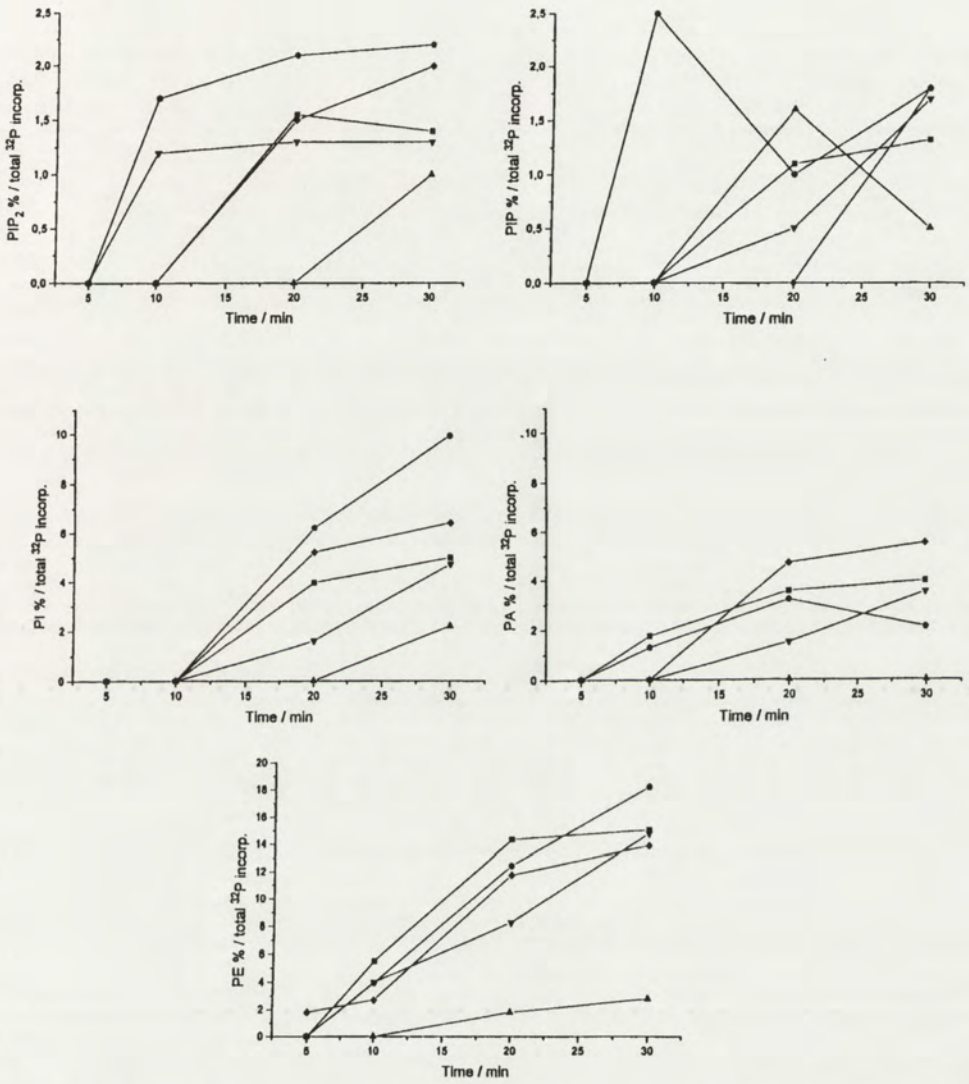

Fig. 1. Incorporation of ^{32}P into the phospholipids of *Tetrahymena*. Effects of single treatments. *Tetrahymena* cultures (25 ml; 10^{-6} cells/ml) were labelled in the logarithmic phase of growth with 12 MBq ^{32}P-sodium orthophosphate (specific activity: 7 GBq/mmol) in absence (control group ■) or presence of 10 mM NaF (●), 20 mM NaF (▲) 10 μM AlCl (▼) and 10 μM $BeCl_2$ (◆). The separation and determination of phospholipids are described in "Materials and Methods". Each point represents the mean value of three parallel experiments

RESULTS

Compared to the product ratios in the control cells, the applied treatments altered the way of incorporation of ^{32}P to the phospholipids of *Tetrahymena* significantly. Treatment with 40 mM NaF totally erased the incorporation of ^{32}P into phosphatidyl acid (PA), phosphatidyl inositol (PI), phosphatidyl-inositol-4-phosphate (PIP), phosphatidyl-inositol-4,5-bis-phosphate (PIP_2) and phosphatidyl-ethanolamine (PE) molecules. Also the 20 mM concentration decreased the incorporation of ^{32}P into the above mentioned molecules except PIP which presented a slight increase. Comparatively, the 10 mM NaF concentration stimulated on the incorporation of ^{32}P into PIP_2, PIP and PI molecules, while labelling of PA and PE was slightly decreased (Fig. 1, Table 1)

The effects of $AlCl_3$ on the ^{32}P incorporation into PIP_2 was similar to the 10 mM NaF, it means that the activity was present earlier (10 min) in the PIP_2 than in the control cells, however the PIP_2 level did not exceed the control level in the subsequent points. $BeCl_2$ essentially resulted in values identical to that of the control.

In the control ^{32}P incorporation into PIP was reduced following the 20 minute incubations with $AlCl_3$ or $BeCl_2$, while the 30 minute treatment with the same substances induced an abrupt increase. There was a

Table 2

Effects of combined treatments on the incorporation of ^{32}P into the phospholipids. Values are means of 3 experiments (% of total incorporated ^{32}P)

Control	PIP_2	PIP	PI	PA	PE
5'	0	0	0	0	0
10'	0	0	0	1.75 ±0.13	5.5 ±0.41
20'	1.55 ±0.08	1.1 ±0.08	3.95 ±0.12	3.55 ±0.22	14.4 ±1.3
30'	1.4 ±0.1	1.32 ±0.12	4.97 ±0.23	3.95 ±0.18	15.15 ±1.18
$AlCl_3$+NaF 10 mM					
5'	0	0	0	0	0
10'	1.7 ±0.1	0	0	0	3.2 ±0.17
20'	1.8 ±0.12	1.3 ±0.1	4.1 ±0.17	1.3 ±0.12	11.6 ±0.9
30'	1.1 ±0.08	1.1 ±0.07	3.2 ±0.09	1.2 ±0.1	6.4 ±0.72
$AlCl_3$+NaF 20 mM					
5'	0	0	0	0	0
10'	0	0	0	0	0
20'	1.4 ±0.07	0	0	0	2.0 ±0.1
30'	1.3 ±0.1	0	0	0	3.2 ±0.07
$BeCl_2$+NaF 10 mM					
5'	1.6 ±0.1	0	0	1.5 ±0.15	2.2 ±0.1
10'	1.8 ±0.09	1.2 ±0.1	0	1.6 ±0.07	3.5 ±0.22
20'	2.7 ±0.17	2.1 ±0.17	9.4 ±0.92	6.3 ±0.43	14.7 ±1.1
30'	2.4 ±0.19	2.3 ±0.16	11.1 ±1.35	7.5 ±0.39	20.9 ±1.35
$BeCl_2$+NaF 20 mM					
5'	0	0	0	0	0
10'	0	0	0	0	0
20'	0	0	0	0	0
30'	0	0	0	0	1.2 ±0.09

similar incorporation into PI following $AlCl_3$ treatment, but $BeCl_2$ increased the ^{32}P content above the control value.

Treatments with $AlCl_3$ and $BeCl_2$ did not result in increased incorporation into the PA and PE at the short incubation times (except the $BeCl_2$ induced incorporation into PE), and the values were about at the control level. The effect of treatment on the incorporation into PA was detectable only later (after 20 min). Following a longer incubation the values were around the control level. The incorporation of ^{32}P into the PA of the $BeCl_2$ treated group was exceptional as its value was considerably higher than the control.

Table 3

Breakdown of ^{32}P-labeled phospholipids (% of total incorporated ^{32}P). The data presented are derived from 3 experiments. Values are means of these experiments.

Control	PIP_2	PIP	PI	PA	PE
0'	3.0 ±0.5	3.6 ±0.4	7.2 ±0.65	9.2 ±0.48	15.0 ±1.2
1'	1.9 ±0.11	3.9 ±0.21	7.9 ±0.8	7.4 ±0.6	18.2 ±1.1
5'	2.0 ±0.17	3.4 ±0.2	7.6 ±0.62	6.7 ±0.31	13.2 ±0.77
15'	1.6 ±0.12	3.5 ±0.16	6.7 ±0.31	6.7 ±0.42	21.5 ±0.65
30'	3.1 ±0.23	8.1 ±0.26	20.4 ±0.18	3.0 ±0.17	51.4 ±2.1
60'	1.2 ±0.14	5.8 ±0.41	24.5 ±1.2	0	36.7 ±1.65
NaF (10mM)					
1'	4.1 ±0.35	5.6 ±0.33	15.8 ±0.82	2.0 ±0.08	41.0 ±2.2
5'	4.4 ±0.28	5.0 ±0.3	11.1 ±0.71	1.5 ±0.1	34.7 ±1.2
15'	6.6 ±0.6	5.9 ±0.16	11.5 ±0.65	1.5 ±0.11	37.2 ±0.8
30'	3.1 ±0.17	7.5 ±0.38	18.5 ±1.0	1.3 ±0.2	41.6 ±1.4
60'	2.2 ±0.17	7.2 ±0.3	26.2 ±0.96	1.5 ±0.07	41.8 ±1.35
NaF+$AlCl_3$					
1'	3.0 ±0.4	3.5 ±0.17	7.0 ±0.8	2.0 ±0.1	27.9 ±1.4
5'	2.5 ±0.16	3.5 ±0.2	9.0 ±0.35	1.3 ±0.07	28.4 ±1.3
15'	6.1 ±0.42	4.2 ±0.32	10.9 ±0.42	1.0 ±0.05	33.2 ±1.88
30'	2.6 ±0.21	7.5 ±0.4	18.5 ±0.77	1.2 ±0.1	40.5 ±2.1
60'	3.7 ±0.28	9.6 ±0.38	26.3 ±0.92	2.0 ±0.07	42.2 ±2.35
NaF+$BeCl_2$					
1'	2.8 ±0.08	4.1 ±0.17	9.4 ±0.33	1.2 ±0.1	32.5 ±1.8
5'	4.7 ±0.19	4.2 ±0.22	5.9 ±0.31	2.2 ±0.08	31.0 ±0.95
15'	5.4 ±0.21	4.4 ±0.16	11.0 ±0.4	1.1 ±0.07	32.3 ±1.3
30'	1.5 ±0.2	5.7 ±0.23	15.5 ±0.63	1.1 ±0.09	30.8 ±1.0
60'	3.1 ±0.17	9.3 ±0.4	33.3 ±1.2	1.5 ±0.09	38.3 ±2.1

Results of the combined treatments, when incubations with NaF and $AlCl_3$ or $BeCl_2$ ran parallel with NaF, presented similar tendency to the single NaF treatments. Treatment with $BeCl_2$ + 10 mM NaF increased significantly the ^{32}P incorporation into PIP_2, PIP and PI molecules. Comparatively, the $AlCl_3$ - NaF combination resulted in significantly increased ^{32}P in-

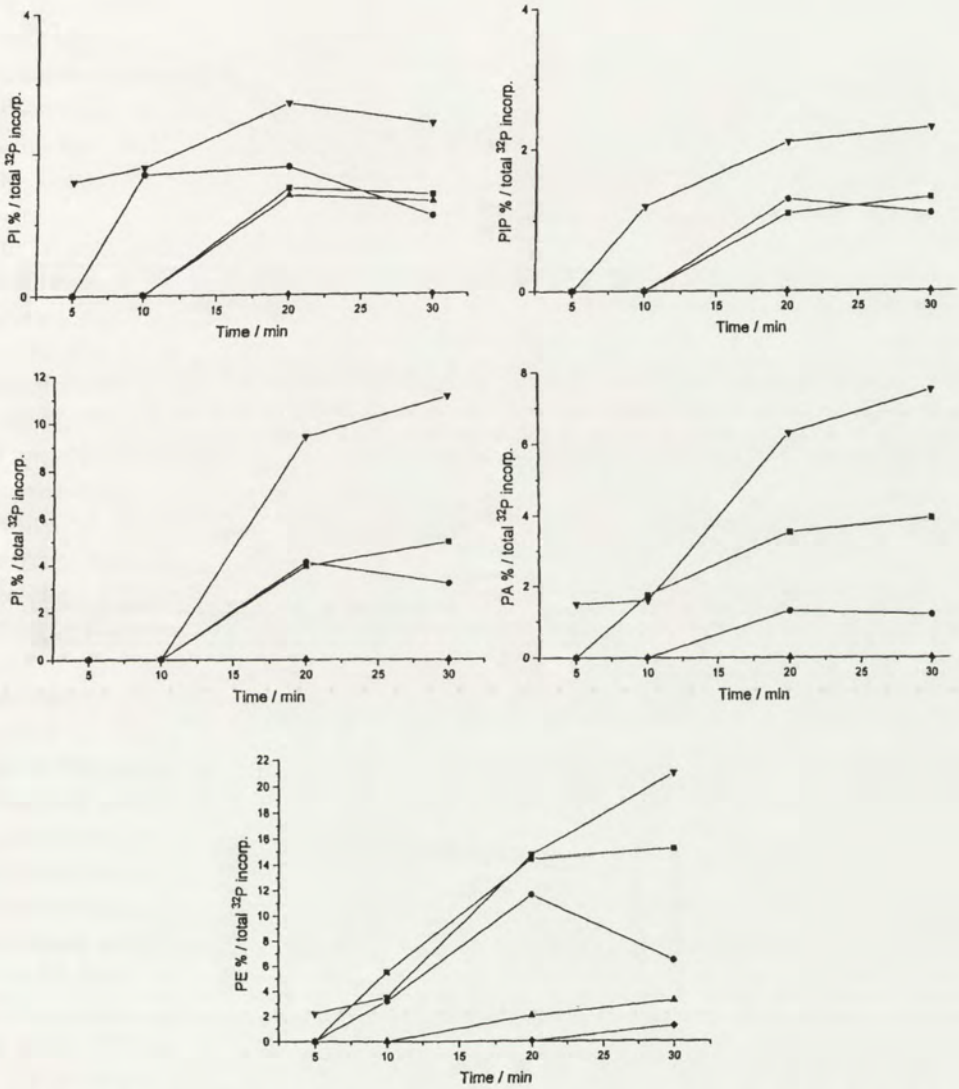

Fig. 2. Incorporation of ^{32}P into the phospholipids of *Tetrahymena*. Effects of combined treatments. Methods as in Fig. 1. ■ - control; ● - $AlCl_3$+NaF 10 mM; ▲ - $AlCl_3$ + NaF 20 mM; ▼ - $BeCl_2$+NaF 10 mM; ◆ - $BeCl_2$+NaF 20 mM

corporation only into the PIP_2. The intensity of incorporation of ^{32}P into PIP and PI was similar to the control at the early times of incubation. Following 30 minutes of incubation there was a decreased percentage ratio of the ^{32}P incorporation into PIP and PI in the control cultures (Fig. 2, Table 2). Applying combinations of 20 mM NaF with $BeCl_2$ or $AlCl_3$ there was no detectable ^{32}P incorporation into the PIP_2, PIP, PI and PA. The only exception was the $AlCl_3$ - NaF combination for PIP_2 when the incorporation was identical to the control value. Combined treatments did not have so drastic effect on the PE. Although there was a decrease of ^{32}P incorporation into PE (except the $BeCl_2$ - NaF 10 mM treatment) neither of the experimental treatments could totally prevent the labelling of PE with ^{32}P.

The investigations of metabolism and breakdown of ^{32}P containing phospholipids, in the treatments with NaF or NaF with $BeCl_2$ or $AlCl_3$ were carried out only at the 10 mM NaF concentration. This was due to the previously noted drastic effects of the higher NaF concentrations. These treatments could also influence significantly the percentage ratios of the studied phospholipids as compared to the total phospholipid content. Following the 1 hour treatment with ^{32}P-Na-orthophosphate, and washing steps, the PIP_2 ratio increased significantly. This elevation of PIP_2 was especially high after 15 minutes but was followed by a decrease at the 30 minute treatment and by another increase after 60 minute (Fig. 3, Table 3).

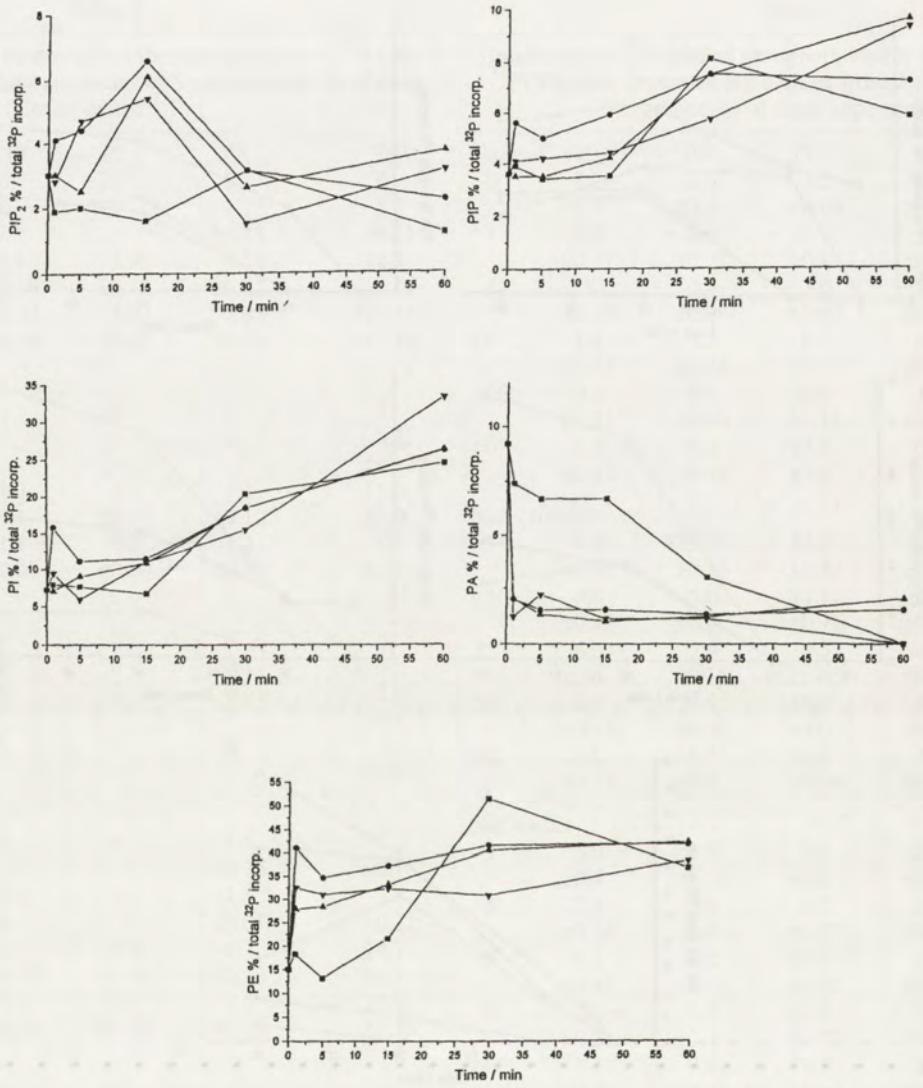

Fig. 3. The breakdown of ^{32}P containing phospholipids after 1h treatment with ^{32}P-Na-orthophosphate. *Tetrahymena* cultures (35 ml; 10^6 cells/ml) were labelled in the logarithmic phase of growth with 20 MBq ^{32}P-sodium orthophosphate (specific activity: 7 GBq/mmol). After 1 hour the cells were washed thrice, and inoculated into fresh medium (35 ml). (0 point of time). After the inoculations NaF (●); NaF+$AlCl_3$ (▲) or NaF+BeCl (▼) were added. Untreated cells served as control (■). At the indicated points of time 5 ml samples were taken and analyzed for phospholipid content

Changes of the PIP and PI ratios presented a similar tendency, but compared to the control *Tetrahymena* group the difference was minimal. The 30 minute treatment was portrayed by equalization between values of the control and the treated groups, then, similarly to the PIP_2 - group, there was an increase after 60 minutes of incubation.

In the case of PA the effect of all of the applied treatments was similar, and there was a fast decrease related to the control which effect was not observed by the 30 minute treatment.

The 30 minute treatment resulted divergent result for PE from the other time points. Up to 15 minute all treatments resulted in a significant increase in ^{32}P incorporation was observed into PE as compared to the control, while following the 30 minute incubation there was a significant decrease which seemed to be equalized by 60 minutes.

DISCUSSION

On the basis of the experimental results we have demonstrated that agents which influence G-proteins (AlF_4 and BeF_3) and NaF which acts also on G-proteins have the capacity to influence significantly the synthesis,

metabolism and breakdown of phospholipids in *Tetrahymena*. These findings are in agreement with our previous experimental results concerning the presence and function of PI system in *Tetrahymena* (Kovács and Csaba 1990) and the production of inositol phospholipids is a G-protein linked mechanism in *Tetrahymena*.

Treatment with 10 mM NaF alone or in combination with $BeCl_2$ or $AlCl_3$ made possible a rapid ^{32}P incorporation and appearance in the members of the PI system (PIP_2, PIP, PI). On the other hand these treatments increased the ratio of the PI system compared to the total phospholipid content. However the higher concentrations of NaF (20 and 40 mM) eliminated the incorporation of ^{32}P into the mentioned phospholipids. This might be due to the ability of NaF to inhibit the activity of certain enzymes (e.g. tyrosine kinases, phosphatases, esterases) (Vinals et al. 1993). At higher doses of NaF this inhibitory effect exceeded the result of G-protein activation.

Studies on the metabolism and breakdown of phospholipids also point to the effect developed by the applied agents on the PI system. Following the treatment with Na-orthophosphate and washing the decrease of the PA content was parallel to the increase in PIP_2, PIP and PI content. This phenomenon could be explained if PA is considered to be a synthetic precursor of the members of the PI system (Sekar and Hokin 1986).

The increase in PE level could be also be explained by the fact that PE is a precursor of PA through the PC molecule (Smith et al. 1992).

The results of the present experiments are insufficient to explain the effect of beryllium and aluminium chlorides on the phospholipids. Indirect or direct effects of the metal ions on the enzymes are possible. However these effects were not found to be identical to the effects of fluorides.

Previously the presence of G-protein was demonstrated in other Protozoa, at the low level of evolutionary development. According to the present experimental results it is probable that G-proteins are also present in *Tetrahymena* and they functions, at least on the PI system, and similar to the functions observed in higher level organisms.

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European Hare and European Rabbit (Lagomorpha) as Intermediate Hosts of *Sarcocystis* Species (Sporozoa) in Central Europe

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Summary. *Sarcocystis cuniculi* Brumpt, 1913 was found in *Oryctolagus cuniculus* from Berlin. This species shows striking features of the cyst wall already under light microscope: the surface is covered by tightly packed slim-looking finger-like villar protrusions. The ultrastructure of the cyst wall is characteristic: intermediate between the types 9 and 10 of the classification by Dubey et al. (1989)(Figs. 1-4). Here the cross-sections of the finger-like villar protrusions are irregularly polygonal. No *Sarcocystis* species has been described in detail and recognizably from *Lepus europaeus*. We found four different species in European hare from Poland. The first (Figs. 5-8) strongly resembles *S. cuniculi*. An ultrastructural feature differs, however: the round outline of the cross-section of the finger-like villar protrusions. Therefore we state this species as *S.* cf. *cuniculi* for the present. The second species from hare (Figs. 9-12) is listed here as "*Sarcocystis* sp. 1". It is characterized by hair-like villar protrusions of the cyst wall as known of one respective species from roe deer, cattle, sheep, goat and horse (type 7 of the classification by Dubey et al. 1989). We designate the third species from hare (Figs. 13-16) as "*Sarcocystis* sp. 2". It shows an ultrastructure of the cyst wall hitherto not yet described, which bears a remote resemblance to the type 21 of the classification by Dubey et al. (1989). The fourth species from hare (Figs. 17-20) is morphologically not discernible from a species hitherto known from equids only, especially on the basis of TEM micrographs of the cyst wall (type 11 of the classification by Dubey et al. 1989). Therefore, we list it as *Sarcocystis* cf. *bertrami* Doflein, 1901 (= *equicanis* = *fayeri*).

Key Words. *Sarcocystis, Oryctolagus, Lepus, Equus*, host specificity.

INTRODUCTION

One *Sarcocystis* species (*S. cuniculi*) from the European rabbit (*Oryctolagus cuniculus*) inclusive the domesticated form is known more detailed since 1976 only (Tadros and Laarman 1976, 1977, 1978, 1982; Černá et al. 1981; and others) among lagomorphs in Europe. The cat serves as definitive host. Only the data by Witzmann (1982) and Witzmann et al. (1983) exist on the occurrence of *Sarcocystis* in the European hare (*Lepus europaeus*) from Thuringia and Brandenburg (Germany). It is not possible to derive anything about the identity of the *Sarcocystis* form(s) from European hare on the basis of this and 19th century statements.

Since 1966, a continuous, year-round study of a hare population has been conducted on experimental hunting grounds at Czempiń (15,000 ha) in western Poland (Pielowski 1994). Every year basic population

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parameters have been determined. A high population level of about 50 individuals/100 ha was maintained by 1970. Its hunting exploitation was about 30% of the autumn stock. When experimentally increased to 40%, it caused a decline in the population density. In the second half of the 1970s, a dramatic population crash took place for reasons not fully understood, and afterwards the population had not recovered, its mean density being maintained at c. 20 individuals/100 ha. Some population parameters were markedly reduced. Hunting exploitation was not responsible for this situation, and it was reduced to a minimum. The basic reason for this permanent depression seems to be a high mortality of adult hares, reaching 30% in the breeding season. The probable causes of this phenomenon can only be environmental pollution and diseases; however, the sarcocysts found in the present investigation can be ignored as acting on the hare population studied.

The aim of the investigations presented here is to analyze the *Sarcocystis* species composition in European rabbit and European hare, in first line in regard to the identification of the forms occurring in hare and their relation to the main form from rabbit.

MATERIALS AND METHODS

Within the scope of a joint German-Polish Hare Investigation Programme (starting in late February, 1993 and ending in early February, 1994) 10 hares each in about monthly intervals were lab-examined for the occurrence of *Sarcocystis*. The hares originated from the hunting district of the Research Station of the Polish Hunting Association in Czempiń (near Poznań). Sarcocysts from free-ranging wild rabbits from the area of the zoo "Tierpark Berlin-Friedrichsfelde" were compared at both the light and electron microscopic levels. Samples of muscle tissue from tongue, diaphragm, oesophagus, heart and skeletal musculature (thigh, loin, thorax, ribs) were investigated, usually in a fresh state. The sarcocysts found in the musculature were extracted from the muscular fibers under a dissecting microscope for fresh-state examination and/or processed for histological and transmission electron microscopical (TEM) investigations. For light level studies, tissues were fixed in 4 % formaldehyde, sections were cut at 3 to 5 µm and stained with hematoxylin and eosin. Using semithin sections or fresh preparations, the size of the bradyzoites (cystozoites) was determined. The length of the bradyzoites was taken by measuring the more or less bent median line from pole to pole. The width was measured at the widest diameter. For TEM investigation the sarcocysts were fixed according to Pospischil and v. Bomhard (1979). After repeated washing with 0.1 molar phosphate buffer, they were post-fixed or pre-contrasted in 2% osmium tetroxide solution, dehydrated in ethanol and embedded in Epon 812. After polymerization for 3 days semithin and ultrathin sections were cut. The semithin sections were stained according to Richardson. The TEM investigations were carried out using a Zeiss EM 902 A.

Abbreviations: $\bar{x}$ - mean value, s - standard deviation, n - number of the elements measured.

RESULTS

One hare of each lot (composed of 10) showed infection with sarcocysts late in March and April, early and late in June, early in November, 1993, and early in February, 1994 (6 from 119 hares examined, - 5 %).

1. *Sarcocystis cuniculi* Brumpt, 1913

Host: *Oryctolagus cuniculus*, agriot.

Locality: Berlin-Friedrichsfelde

Site: Muscle fibers around oesophagus, tongue, ribs, thorax, diaphragm, thigh and loin

Frequency: 3 of 4 examined rabbits infected, intensity high (at least 500 sarcocysts in each rabbit)

Description (Table 1, Figs. 1-4). Cyst wall with tightly packed long slim-looking finger-like villar protrusions. These showed a slightly tapering distal portion with rounded top and were a little narrowed at their base. The villar protrusions had mostly a polygonal outline in the ultrathin cross-section (in a few cases, however, also a round outline). In the core of the villar protrusions lay microtubules running from the top as far as into the ground substance of the cyst wall. Sometimes it was recognizable that the microtubules changed their direction after reaching the ground substance, thus running a short distance parallel to the cyst wall. The microtubules were distributed evenly on the cross-section of the villar protrusions, but either the extreme edges of the outline of the cross-sections remained free or the whole range of the microtubules was surrounded by an electron-pale zone. This marginal zone was only occasionally recognizable on longitudinal sections.

Measurements: diameter of the microtubules 22-25 nm. Distances (diameter) of the compartments in the region of the cyst wall 11.4-29.9 µm ($\bar{x}$ = 20.9 µm, s = 4.4, n = 30); depth of the compartments in direction of the centre 11.4-22.3 µm ($\bar{x}$ = 17.5 µm, s = 3.1, n = 10).

2. *Sarcocystis* cf. *cuniculi* Brumpt, 1913

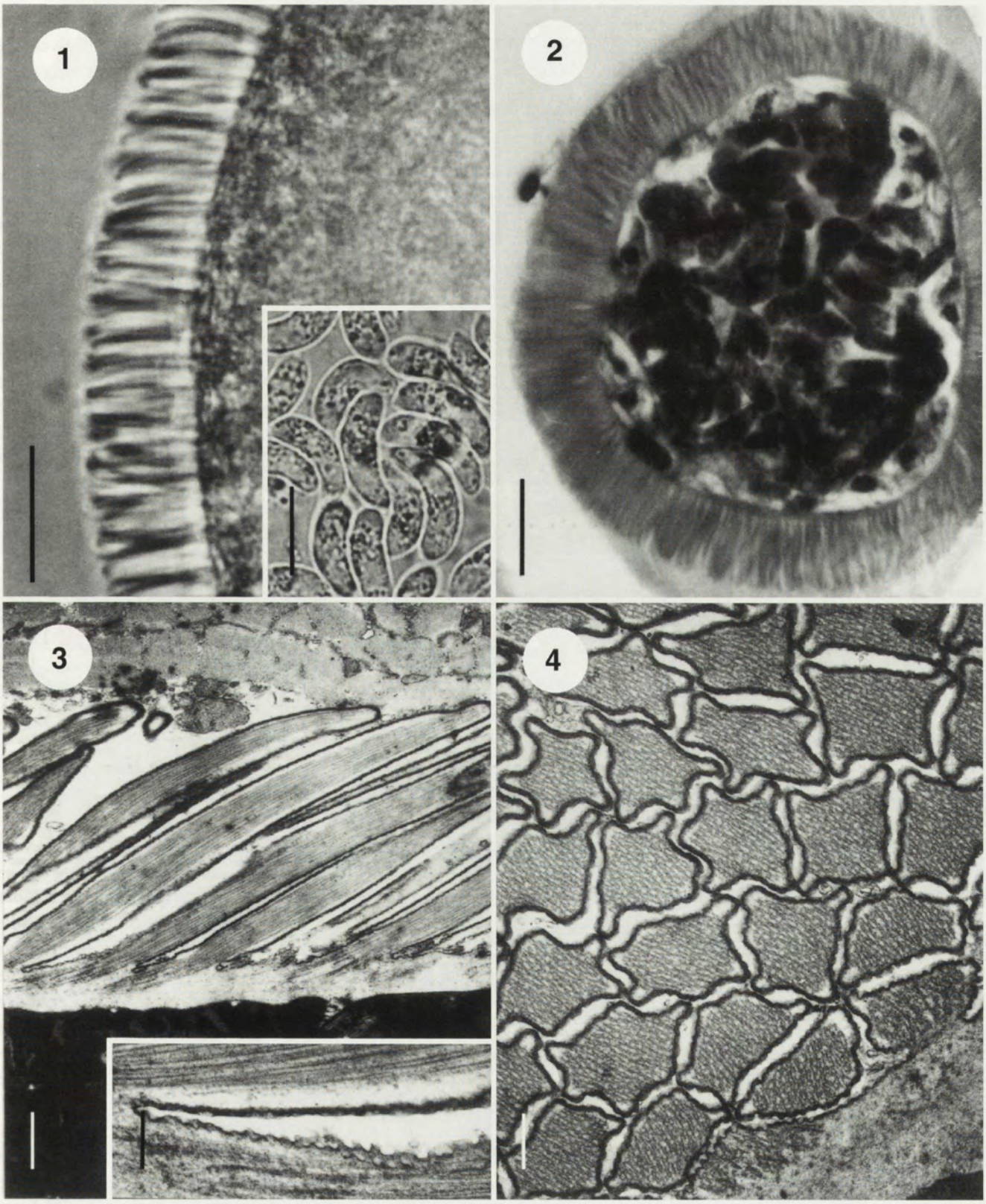
Host: *Lepus europaeus*

Locality: Hunting district Czempiń (near Poznań)

Site: Muscle fibers of diaphragm, thigh and loin

Frequency: 2x, intensity slight

Description (Table 2, Figs. 5-8). Cyst wall with tightly packed long slim-looking finger-like villar



Figs. 1-4. *Sarcocystis cuniculi* from rabbit. 1 - part of the palisade-like cyst wall in a fresh preparation; inset: free bradyzoites in the fresh state (all bars - 10 µm). 2 - cross-section of a sarcocyst, bar - 10 µm
3-4. TEM micrographs of the wall of a 1.6 mm long mature sarcocyst (same as in Fig. 2) from rib muscle. 3 - longitudinal section of the finger-like protrusions, bar - 1.1 µm; inset: detail of the invaginated surface and the microtubules of a finger-like protrusion, bar - 0.4 µm. 4 - cross-section of the finger-like protrusions (note the polygonal outline), bar - 0.6 µm

protrusions. These showed a slightly tapering distal portion with rounded top and were a little narrowed at their base. The villar protrusions were always round to oval in outline on the ultrathin cross-section. In the core of the villar protrusions lay microtubules running from the top as far as into the ground substance of the cyst wall. Sometimes it was recognizable that the microtubules changed their direction after reaching the ground substance, thus running a short distance parallel to the cyst wall. The microtubules were distributed evenly on the cross-section of the villar protrusions, but concentrated in the centre. A broad electron-pale margin surrounded the central region of the microtubules (about 61% of the total cross-section on average). This marginal region without microtubules could also be distinctly seen on the longitudinal sections of the villar protrusions.

Measurements: diameter of the microtubules 22-25 nm. Distances of the compartments in the region of the cyst wall 25.7-54.3 µm ($\bar{x}$ = 39.2 µm, s = 8.3, n = 15); depth of the compartments in direction of the centre 25.7-50.1 µm ($\bar{x}$ = 37.2 µm, s = 8.2, n= 10).

3. *Sarcocystis* sp. 1

Host: *Lepus europaeus*
Locality: Hunting district Czempiń (near Poznań)
Site: Muscle fibers of heart and thighs
Frequency: 2x, intensity slight

Description (Table 3, Figs. 9-12). Cyst wall at irregular distances (2.9-9.7 µm) with unstable hair-like villar protrusions occurring singularly or sometimes in more or less closely packed groups. The interior of the villar protrusions was interspersed with numerous fine and a few larger granules. Different parts of the hair-like villar protrusions were mostly not recognizable, but they tapered continuously up to the top. In some cases it could be seen that they arose from dome-shaped bases on the primary cyst wall.

Measurements: distances of the compartments in the region of the cyst wall: 13.3-114.4 µm ($\bar{x}$ = 46.5, s = 24.2, n = 30); depth of the compartments in direction of the centre: 18.3-99.7 µm ($\bar{x}$ = 41.5, s = 22.6, n = 10).

4. *Sarcocystis* sp. 2

Host: *Lepus europaeus*
Locality: Hunting district Czempiń (near Poznań)
Site: Muscle fibers around oesophagus and trachea
Frequency: 1x, intensity slight

Description (Table 4, Figs. 13-16). Cyst wall with irregularly shaped and much ramified cauliflower-like villar protrusions with undulated margins, arising from the surface of the cyst with a relatively thin and short stalk. The lobules on the undulated margins were 0.15-0.21 µm high and 0.15-0.18 µm wide on TEM micrographs, the respective invaginations were 0.05-0.14 µm deep and 0.11-0.17 µm wide. Microtubules ran in the core of the villar protrusions, appearing to be interrupted on the section, and large granules lay obviously arranged in rows. In the distal half of the ground substance a row of large granules ran parallel to the cyst surface.

Measurements: the distance of the compartments in the region of the cyst wall was relatively regular on the semithin section (apart from the apical region of the cyst): 26.6-36.6 µm ($\bar{x}$ = 31.8 µm, s = 4.1, n = 20). Depth of the compartments in direction of the centre of the cyst 28.4-49.7 µm ($\bar{x}$ = 43.4 µm, s = 12.7, n = 20).

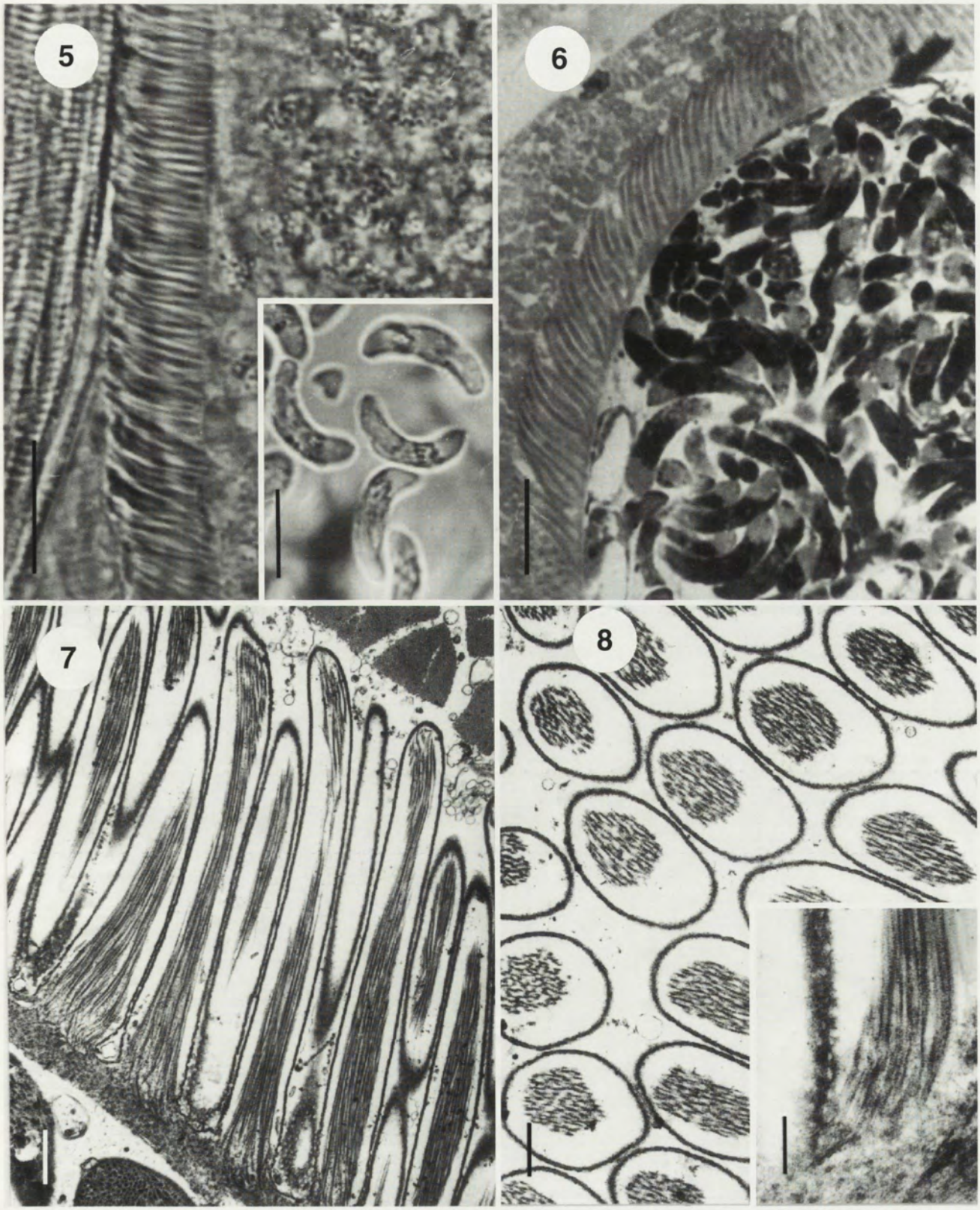
5. *Sarcocystis* cf. *bertrami* Doflein, 1901

Synonyms of *S. bertrami: S. equicanis* Rommel and Geisel, 1975 (according to Hinaidy and Loupal 1982); *S. fayeri* Dubey Streitel, Stromberg and Toussant, 1977 (according to Hinaidy and Loupal 1982); S. sp. Hilali and Nassar, 1989

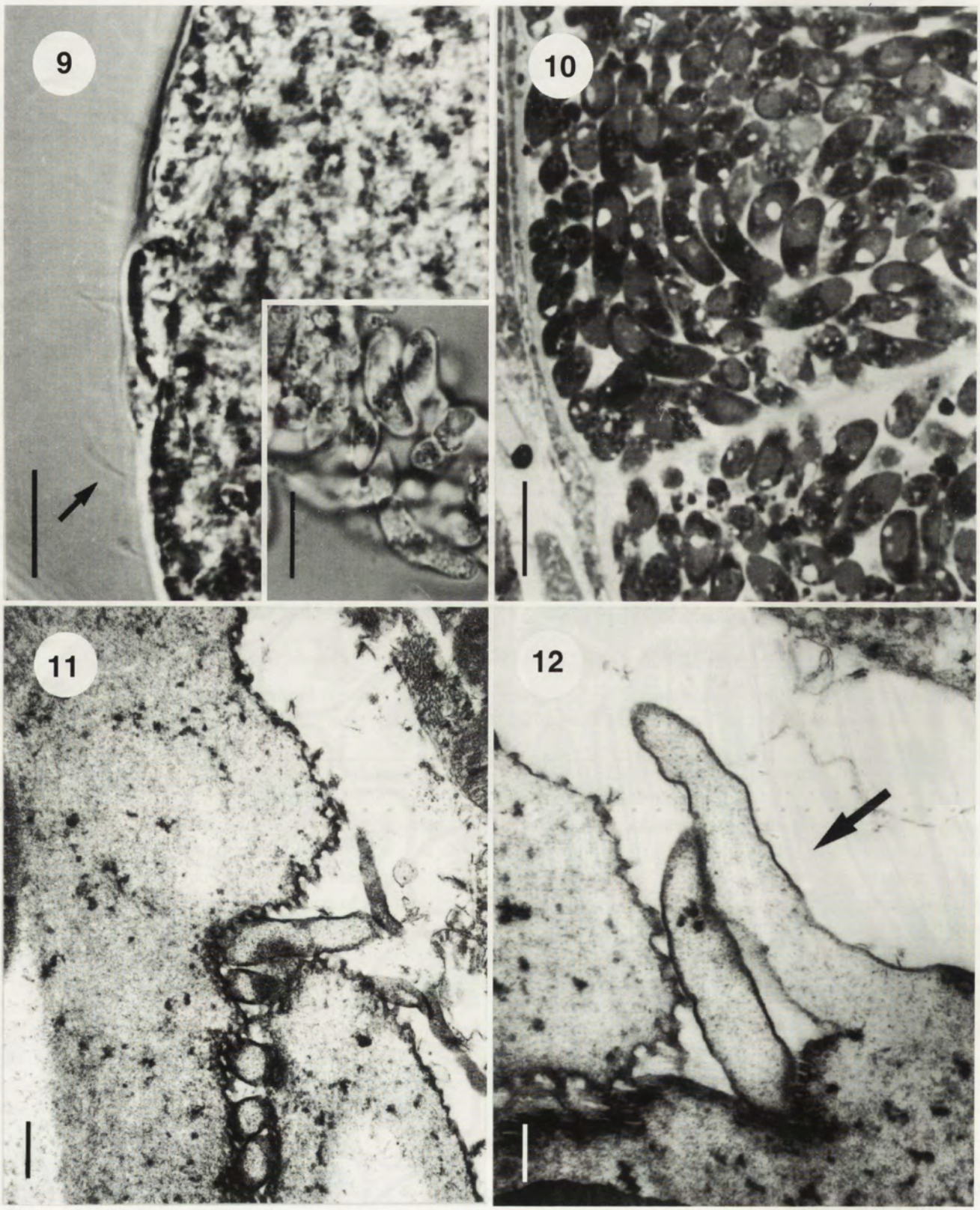
Host: *Lepus europaeus*
Locality: Hunting district Czempiń (near Poznań)
Site: Muscle fibers of loin
Frequency: 1x, intensity slight

Description (Table 5, Figs. 17-20). Cyst wall with elongated leaflet-like villar protrusions arising at irregular, mostly rather spacious distances of each other from the surface of the cyst and often clinging to it like scales. The undulating margins of the villar protrusions were generally coarser than the primary cyst wall by its invaginations between the villar protrusions. A dense bundle of microtubules lay in the core of the villar protrusions, penetrating deeply into the ground substance in continuation of the longitudinal axis of the villar protrusion. Coarse osmiophilic granules in different numbers lay around the bundle of microtubules.

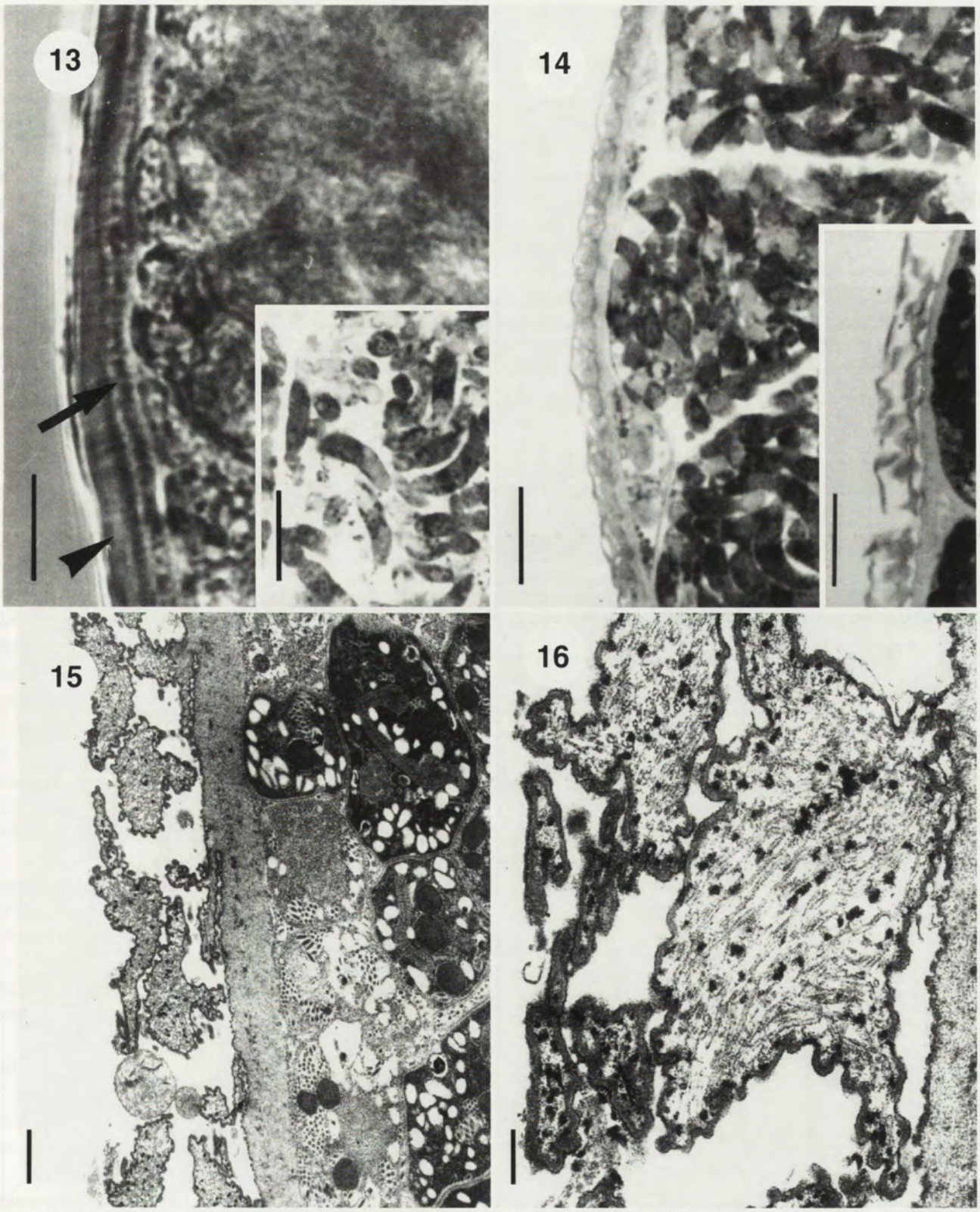
Measurements: diameter of the microtubules 22-28 nm ($\bar{x}$ = 24 nm, s = 0.002, n = 10). Distances of the compartments in the region of the cyst wall 7.7-20.1 µm ($\bar{x}$ = 13.2 µm, s = 4.2, n = 20). Depth of the compartments in direction of the centre of the cysts 12.9-22.9 µm ($\bar{x}$ = 18.0, s = 2.8, n = 15).



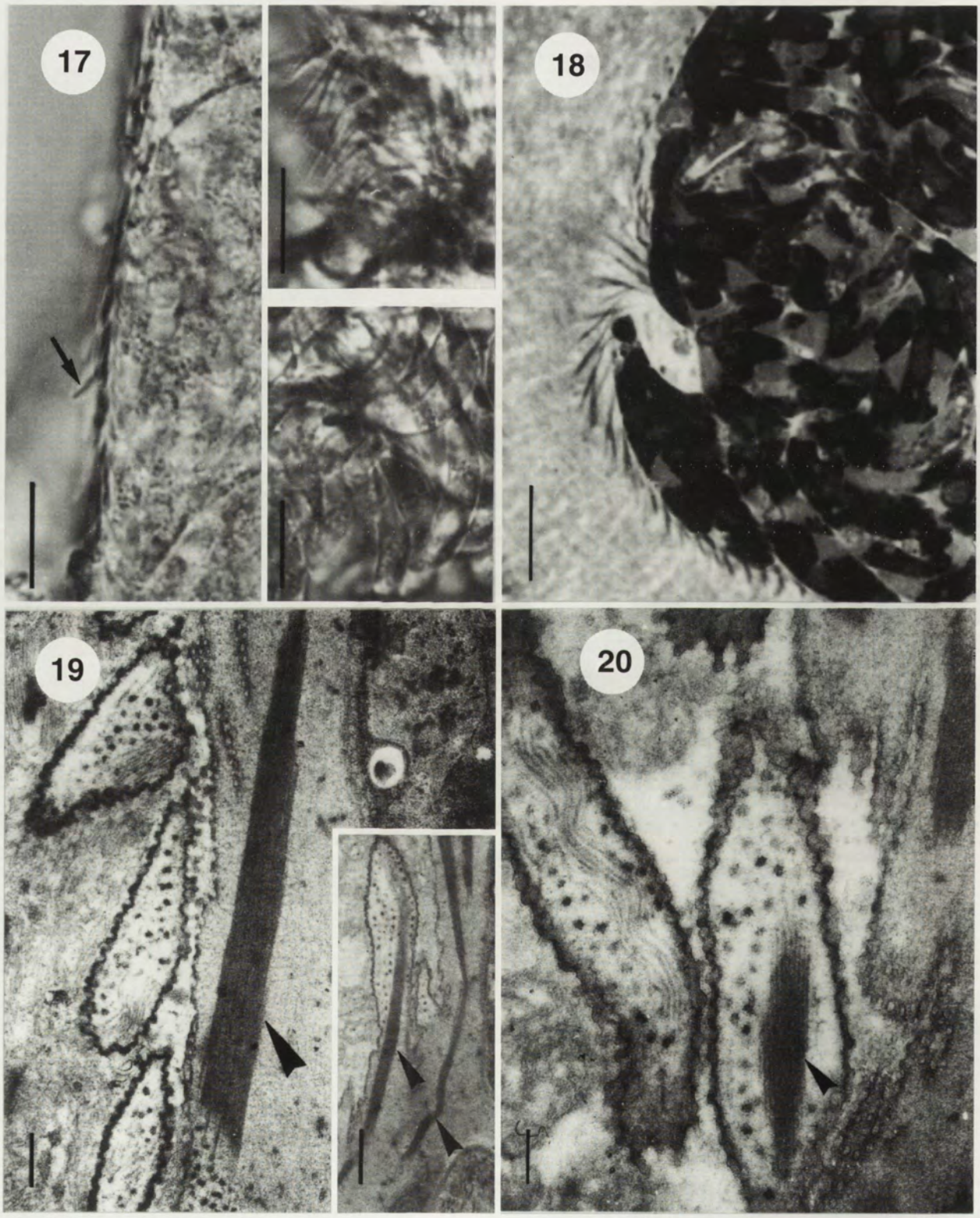
Figs. 5-8. *Sarcocystis* cf. *cuniculi* from hare. 5 - part of the palisade-like cyst wall in a fresh preparation; inset: free bradyzoites in the fresh state (all bars - 10 µm). 6 - semithin cross-section of a sarcocyst, bar - 10 µm
7-8. TEM micrographs of the wall of a 2 mm long mature sarcocyst (same as in Fig. 6) from loin muscle. 7 - longitudinal section of the finger-like protrusions, bar - 1.1 µm. 8 - cross-section of the finger-like protrusions (note the oval outline), bar - 0.6 µm; inset: detail of the invaginated surface and the microtubules of a finger-like protrusion, bar - 0.4 µm



Figs. 9-12. *Sarcocystis* sp. 1 from hare. 9 - part of a sarcocyst with hair-like protrusions (arrow) of the cyst wall in a fresh preparation; inset: free bradyzoites in the fresh state (all bars - 10 µm). 10 - semithin cross-section of a sarcocyst, bar - 10 µm.
11-12. TEM micrographs of the wall of a 1.1 mm long mature sarcocyst (same as in Fig. 10) from heart muscle. 11 - diagonal and cross-sections of the hair-like protrusions, bar - 0.6 µm. 12 - proximal part of two hair-like protrusions (arrow) in longitudinal section, bar - 0.4 µm



Figs. 13-16. *Sarcocystis* sp. 2 from hare. 13 - part of a sarcocyst in a fresh preparation, surrounded by its host cell remnant (arrowhead), beneath it the villar protrusion layer of the cyst wall (arrow); inset: free bradyzoites in the fresh state (all bars - 10 µm). 14 - part with three compartments of a semithin cross-section of a sarcocyst; inset: detail of the cyst wall (of the same cyst), showing the cauliflower-like protrusions (all bars - 10 µm)
15-16. TEM micrographs of the cyst wall of a 4.3 mm long mature sarcocyst (same as in Fig. 14) from muscle around trachea. 15 - complete cyst wall with adjacent core, bar - 1.1 µm. 16 - detail of villar protrusions, bar - 0.4 µm



Figs. 17-20. *Sarcocystis* cf. *bertrami* from hare. 17 - part of a sarcocyst with elongated leaflet-like protrusions (arrow) in a fresh preparation; upper inset: detail of the cyst wall surface in the fresh state; lower inset: bradyzoites in fresh state (all bars - 10 µm). 18 - semithin cross-section of a sarcocyst, showing the villar protrusions of the cyst wall, bar - 10 µm
19-20. TEM micrographs of the wall of a 4 mm long mature sarcocyst (same as in Fig. 18) from loin muscle. 19 - cyst wall with three interrupted leaflet-like protrusions and the continuation within the ground substance of the bundle of microtubules of another protrusion (arrowhead), bar - 0.6 µm; inset: uninterrupted protrusions showing the microtubules running from core of the protrusions into granular layer (arrowheads), bar - 1.1 µm. 20 - detail of the protrusions with microtubules (arrowhead) and large osmiophilic granules, bar - 0.4 µm

DISCUSSION

As early as 1867 Manz mentioned "Mieschersche Schläuche" in the "rabbit" (*Oryctolagus cuniculus*) from south-west Germany. Tadros and Laarman (1976: *Sarcocystis* sp., 1977: *S. cuniculi* Brumpt, 1913) rediscovered sarcocysts in the wild European rabbit (*O. cuniculus*, agriot.) in the Netherlands and established the cat as definitive host. Tadros and Laarman (1978, 1982) published thorough light and electron microscopic descriptions of *S. cuniculi*. This species occurs also in the domestic rabbit (*O. cuniculus*, hemerot.) in Europe (findings and description from Bohemia as well as immunological work and experimental elaboration of the life cycle by Černá et al. 1981, cf. also Černá 1983; further findings and descriptions by Lukešová et al. 1984 from Moravia and by Donát 1989 from Bohemia, Czech Republic). Elwasila et al. (1984) described *S. cuniculi* from wild rabbits (*O. cuniculus,* agriot.) in the Rhine region (Bonn area, Germany). Unger (1977) mentioned *Sarcocystis* infections, undetermined in detail, in *O. cuniculus,* agriot. from Land Brandenburg (Germany). *S. cuniculi* occurs apart from Europe also in Australia and New Zealand in *O. cuniculus*, agriot. (cf. Collins 1979, Munday et al. 1980). Tadros and Laarman (1982) stated that a further, rare *Sarcocystis* species with thin cyst wall occurs in *O. cuniculus*, agriot. from the Netherlands.

The material from *O. cuniculus*, agriot. examined by us corresponds well with the hitherto existing light and electron microscopic descriptions in all essential features and measures mainly of the cyst wall diagnostically important for the species:

- the long finger-like villar protrusions striking already by light microscopy (Tadros and Laarman 1976, 1978, 1982); Černá et al. 1981; Černá 1983; Donát 1989);
- the change of the course of the microtubules from the villar protrusions into the ground substance (Tadros and Laarman 1978, Elwasila et al. 1984);
- the polygonal outline of the cross-sections of the villar protrusions (Elwasila et al. 1984).

Dubey et al. (1989) put *S. cuniculi* to their ultrastructural wall type 10 (a type to which belong such well known species as *S. hirsuta* and *S. hominis* from cattle, but also *S. leporum* to which we shall return below). This attribution is not very clear and shows the blunt separation of the types 9 and 10: type 9 has villar protrusions more distant of each other and microtubules reaching into the ground substance, whereas type 10 has closely packed villar protrusions and microtubules not reaching into the ground substance. *S. cuniculi* therefore unites features of type 9 (microtubules reaching into the ground substance) and type 10 (tightly packed villar protrusions).

We list the form found by us in the hare and bearing great similarity to *S. cuniculi* provisionally as *S.* cf. *cuniculi* because the cross-sections of the villar protrusions are different on the TEM micrographs (predominantly polygonal in *S. cuniculi*, round in the form from hare, cf. Figs. 4 and 8). Whereas these cross-sections were always round in the form from hare the micrographs of *S. cuniculi* sometimes had a transitional appearance. We think it most likely that the form from hare is identical with *S. cuniculi*. This should, however, be corroborated by further investigations. One should also think of the possibility of host modifications (though not known in sarcocysts yet).

There are inconsistent statements in the literature on the identity of the species *S. cuniculi* and *S. leporum. S. leporum* was described from the cottontail rabbit (*Sylvilagus*) in North America (Crawley 1914). Despite the fact that *S. leporum* was described in 1914, Babudieri (1932) retained that name and synonymized *S. cuniculi* with it though it had been described a year earlier, an obvious violation of the principles of zoological nomenclature. He was followed by Kalyakin and Zasukhin (1975) in this alleged identity, but in the correct sequence: *leporum* as a synonym of *cuniculi*. Most American authors, however, did not adopt this synonymy (e. g., Erickson 1946, Vande Vusse 1967, Crum and Prestwood 1977, Fayer and Kradel 1977), neither did Levine and Tadros (1980), Levine (1986, 1988) nor Dubey et al. (1989). The life cycle of *S. leporum* was experimentally elucidated by Fayer and Kradel (1977) and by Crum and Prestwood (1977). In both cases the cat proved to be the definitive host. Crum and Prestwood (1977), however, reported in an addendum to their paper that also the raccoon can serve as definitive host and would be even more appropriate than the cat. This could indicate that there are two different *Sarcocystis* species in *Sylvilagus*. Cosgrove et al. (1982) designated their findings of sarcocysts in *Sylvilagus floridanus* merely as "*Sarcocystis* sp.". Munday et al. (1980) and Elwasila et al. (1984) compared the sarcocysts of *S. cuniculi* and *S. leporum*. Both teams claimed that there were no or only minor morphological differences. Such differences are, however, quite distinctly recognizable in the ultrastructure in our opinion, at least in the data by Elwasila et al. (1984) on the ultrastructure:

- the villar protrusions of the cyst wall are 5-6 µm long in *S. leporum* and 8-12 µm long in *S. cuniculi*;

- in *S. leporum* granules and bundles of fibrillar elements lie in the interior of the villar protrusions; the fibrillar elements do not appear continuously (on the micrographs) nor do they penetrate the ground substance. In *S. cuniculi* granules are lacking in the interior of the villar protrusions, and microtubules run continuously from the top as far as into the ground substance where they change their direction;

- the ground substance (= granular layer) is up to 3 µm thick in *S. leporum*, but maximum 1.5 µm in *S. cuniculi*.

Besides, experiments were done in which domestic rabbits, *O. cuniculus,* hemerot., were infected with sporocysts of *S. leporum*, but no sarcocysts were found in the tissues (Fayer and Kradel 1977). In addition, because of the distinct ultrastructural differences between *S. cuniculi* and *S. leporum,* it is clear that they are separate species.

Further *Sarcocystis* species were described from species of the genus *Ochotona* (piping hares) in Siberia and Kazakhstan. *Sarcocystis dogeli* Machul'skii, 1947 was found in *Ochotona daurica* from the northern shore of lake Baikal. Its sarcocysts are bagshaped and measure 0.8-1.2 mm x 0.4-0.5 mm according to Machul'skii (1947). There are two reports from Kazakhstan, in each case on two *Sarcocystis* species in *Ochotona alpina* (Levit et al. 1984, Fedoseenko 1986). *Sarcocystis galuzoi* Levit et al., 1984 occurs within cells of connective tissue and forms oval, 0.5-2.0 mm long sarcocysts with a "smooth", 3-5 µm thick wall. *S.* sp. Levit et al., 1984 forms thread-like, 0.9-3.5 mm long sarcocysts with a "smooth", 0.5-1.5 µm thick cyst wall within muscle cells. *S.* sp. 1 Fedoseenko develops round macrocysts with a diameter of up to 3 mm within muscle cells, where also the thread-like microcysts of *S.* sp. 2 Fedoseenko, 1986 occur. The descriptions of these four species available to us only allow the statement that the species *S. galuzoi* and *S.* sp. 1 Fedoseenko do not correspond with the other forms from lagomorphs (inclusive our findings) because of the localization and/or shape.

We cannot proceed on the assumption that all the *Sarcocystis* species found in hare are specific parasites of lagomorphs or even merely of *Lepus europaeus*, considering that of four different *Sarcocystis* species recently found in badger (*Meles*) only one has been believed to be specific for badger, two others, however, have been recognized as species most likely or really typical for roe deer (*Capreolus*) (cf. Odening et al. 1994a, b).

The species designated here as "*Sarcocystis* sp. 1" bears some resemblance to *S. capreolicanis* (roe deer), *S. cruzi* (cattle and other Bovinae), *S. arieticanis* (sheep), *S. hircicanis* (goat) and *S.* sp. Yamada et al., 1993 (horse, Japan). The cyst wall belongs to type 7 of the ultrastructure according to the classification by Dubey et al. (1989). All these species are characterized by hair-like villar protrusions of the cyst wall.

Our "*Sarcocystis* sp. 2" shows an ultrastructure of the cyst wall hitherto not described. It bears a remote resemblance to type 21 of the classification of the cyst wall ultrastructure by Dubey et al. (1989) as it is known for *S. gigantea* (macrocysts in sheep) and which applies also to *S. fusiformis* (macrocysts in *Bubalus*). *S. moulei* (macrocysts in goats, cf. Ghaffar et al. 1989) and *S. aucheniae* (macrocysts in llamas, cf. Schnieder et al. 1984) show a more remote similarity.

The fourth species from the hare cannot be distinguished morphologically from *S. bertrami* Doflein, 1901 (sensu Hinaidy and Loupal 1982) on the basis of the corresponding ultrastructure. It is noteworthy that this species has been found hitherto in equids only (the dog is the definitive host). For the synonymous forms the ultrastructure was depicted by Göbel and Rommel (1980) in *S. equicanis* from horses in Germany and by Tinling et al. (1980) in *S. fayeri* from a horse in the USA. Sarcocysts from horse and two species of zebras in Africa showed the same ultrastructure (Daly et al. 1983). Hilali and Nassar (1987) published a further corresponding TEM picture of sarcocysts from donkeys in Egypt. We conceive this, therefore, also as a synonym of *S. bertrami*. The ultrastructures of the cyst wall of all these forms from equids as well as of the form found by us in hare correspond well. They belong to type 11 of the classification by Dubey et al. (1989). Hinaidy and Loupal (1982) performed the synonymy of *S. equicanis* and *S. fayeri* with *S. bertrami* on the basis of a careful and comprehensive analysis of the literature and of extensive own data of examination with light microscope. Additional work needs to be done to prove the identity of our fourth species from the hare with a species of the horse. The only evidence presented here is that they are ultrastructurally very similar. - The often "interrupted" appearance of the microtubules on ultrathin sections could be attributed to their winding course (Fig. 20).

A recently described *Sarcocystis* species from horses in Japan was characterized as resembling "more *S. equicanis* than any other of the described species infective to horse" (Yamada et al. 1993), however, that *Sarcocystis* sp. has a cyst wall which belongs to type 7 of the classification by Dubey et al. (1989) (whereas *S. bertrami* = *equicanis* = *fayeri* represents type 11).

APPENDIX

Table 1

Sarcocysts of *Sarcocystis cuniculi* from *Oryctolagus cuniculus*, measurements

	x̄(μm)	s	n	from (μm)	up to (μm)
Sarcocysts					
Length a)	1605	924	53	399	4551
Width a)	172	34.4	53	40	228
Entire cyst wall					
Width a)	9.2	1.3	35	7.4	11.4
Width b)	6.7	0.9	18	5.5	7.7
Ground substance + PCW b)	1.0	0.1	18	0.8	1.1
Layer of the protrusions b)	5.6	1.1	18	4.4	6.8
Protrusions					
Individual length a)	9.9	1.2	35	7.7	11.7
Individual length b)	10.4	0.8	18	9.4	11.7
Diameter basis b)	1.6	0.3	18	1.1	2.0
Middle b)	1.2	0.4	100	0.7	2.1
PCW invaginations					
Depth b)	0.05	0.006	18	0.04	0.05
Width b)	0.06	0.01	18	0.05	0.08
PCW "small elevations"					
Height b)	0.05	0.007	18	0.04	0.06
Width b)	0.07	0.012	18	0.06	0.09
Bradyzoites					
Length a)	14.0	1.1	40	11.7	15.4
Max. width a)	3.5	0.3	40	2.9	4.0

Abbreviations:
a) – in the fresh state, b) – from TEM micrographs, PCW – primary cyst wall

Table 2

Sarcocysts of *Sarcocystis* cf. *cuniculi* from *Lepus europaeus*, measurements

	x̄(μm)	s	n	from (μm)	up to (μm)
Sarcocysts					
Length a)	1864	227	5	1520	2200
Width a)	149	80	5	74	300
Entire cyst wall					
Width a)	9.3	0.6	45	8.6	10.3
Width b)	8.9	0.6	30	8.0	10.0
Ground substance + PCW b)	0.8	0.13	30	0.5	1.0
Layer of the protrusions b)	8.9	0.16	30	8.8	9.3
Protrusions					
Individual length a)	10.0	0.8	45	8.6	10.9
Individual length b)	10.1	1.0	30	8.0	10.6
Diameter basis b)	1.5	0.3	30	1.1	1.9
Middle b)	1.4	0.2	130	1.0	1.9
PCW invaginations					
Depth b)	0.07	0.014	25	0.06	0.1
PCW "small elevations"					
Height b)	0.07	0.014	25	0.06	0.1
Width b)	0.08	0.009	25	0.06	0.08
Bradyzoites					
Length a)	15.6	0.9	100	13.7	17.7
Max. width a)	3.2	0.33	100	2.7	4.0

Abbreviations as in Table 1

Table 3

Sarcocysts of *Sarcocystis* sp. 1 from *Lepus europaeus*, measurements

	x̄(μm)	s	n	from (μm)	up to (μm)
Sarcocysts					
Length a)			4	105	800
Width a)			4	34	300
Cyst wall without protrusions					
Width a)	1.6	0.52	30	0.9	2.7
Width b)	3.7	2.3	20	0.7	10.0
Protrusions					
Individual length a)	4.5	1.7	10	4.0	6.3
Diameter					
Basis b)	0.5	0.08	20	0.32	0.6
Middle b)	0.23	0.12	20	0.17	0.4
PCW invaginations					
Depth b)	0.12	0.017	10	0.11	0.16
Width b)	0.08	0.005	10	0.07	0.09
PCW "small elevations"					
Height b)	0.12	0.017	10	0.11	0.16
Width b)	0.06	0.006	10	0.05	0.08
Bradyzoites					
Length a)	14.6	0.6	16	13.1	15.4
Max. width a)	4.0	0.33	16	3.4	4.6

Abbreviations as in Table 1

Table 4

Sarcocysts of *Sarcocystis* sp. 2 from *Lepus europaeus*, measurements

	x̄(µm)	s	n	from (µm)	up to (µm)
Sarcocysts Length a)			1		(4342)
Width a)			1		(251)
Entire cyst wall					
Width a)	3.2	0.6	30	2.3	4.0
Width b)	3.0	0.6	13	1.9	3.8
Ground substance + PCW b)	1.0	0.3	13	0.6	1.6
Layer of the protrusions b)	1.7	0.5	13	1.1	2.6
Protrusions					
Individual length b)	1.7	0.5	13	1.1	2.6
Diameter basis b)	0.33	0.14	13	0.11	0.5
Middle b)	3.7	1.2	13	1.3	5.8
Lenght of the stalk b)	0.23	0.08	13	0.11	0.4
PCW invaginations					
Depth b)	0.05	0.009	13	0.04	0.05
Width b)	0.03	0.01	13	0.03	0.05
PCW "small elevations"					
Height b)	0.05	0.006	13	0.04	0.05
Width b)	0.06	0.008	13	0.05	0.08
Bradyzoites					
Length a)	14.9	1.6	30	12.6	17.1
Max. width a)	3.5	0.18	30	3.4	4.0

Abbreviations as in Table 1

Table 5

Sarcocysts of *Sarcocystis* cf. *bertrami* from *Lepus europaeus*, measurements

	x̄(µm)	s	n	from (µm)	up to (µm)
Sarcocysts					
Length a)			2	1450	4000
Width a)			2	82	160
Entire cyst wall					
Width a)	3.2	0.8	30	1.9	4.4
Width b)	2.5	0.6	30	1.7	3.6
Ground substance + PCW b)	1.1	0.37	30	0.7	1.4
Layer of the protrusions b)	1.1	0.28	30	0.8	1.7
Protrusions					
Individual length a)	6.3	0.6	20	5.6	7.6
Individual length b)	4.9	0.7	20	3.5	5.5
Diameter					
Basis (lateral view) b)	1.0	0.23	10	0.6	1.2
Middle b)	0.8	0.14	15	0.5	1.1
Distances between the protrusions b)	3.7	0.7	10	2.8	4.6
PCW invaginations					
Depth b)	0.04	0.011	20	0.02	0.06
Width b)	0.05	0.018	20	0.04	0.09
Bradyzoites					
Length a)	17.4	0.4	15	16.9	17.8
Length b)	13.1	2.5	10	10.0	17.5
Length in semithin section	14.9	1.4	30	12.9	17.6
Max. width a)	3.7	0.4	15	3.1	4.4
Max. width b)	3.3	0.5	10	2.5	3.9
Max. width in semithin section	3.0	0.3	30	2.6	3.5

Abbreviations as in Table 1

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Short communication

Experimental Transmission of Murine Malaria by Cannibalism on Mice Infected by *Plasmodium berghei yoelii*

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Summary. To demonstrate the existence of malaria transmission by cannibalism, six experiments were conducted. A total of 108 young male CD1 mice were kept unfed for about 12h and then each experimental mouse, caged separately, was offered overnight with the corpse of a freshly sacrificed mouse infected with *Plasmodium berghei yoelii*, while each of the control mice was offered with the corpse of a clean mouse. All mice were subjected to detection of malaria infection by blood smears stained with giemsa. Mice fed with corpses of infected mice acquired the infection in 35% of cases. No control mice acquired the infection. The approximate prepatency of infection ranged from 1 to 3 weeks. It is demonstrated, in a rodent model, that malaria infection may be transmitted among vertebrate hosts of the same species in absence of mosquitos, by eating flesh and organs of malaria infected animals.

Key words. *Plasmodium berghei*, rodent malaria, oral transmission, transmission by cannibalism, experimental.

INTRODUCTION

Natural transmission of human malaria occurs in areas where at least three conditions are fulfilled: a summer temperature of no less than 16°C, presence of *Anopheles* mosquitos, and human beings infected with malaria parasites (Bruce-Chwatt 1980).

The transmission take place when infected *Anopheles* mosquitos introduce sporozoites into the blood of a human host, while biting. Sporozoites grow in the liver and produce forms that invade blood cells, where they grow cyclicly by asexual fashion and produce, at the same time, sexual forms able to infect mosquitos when feeding on blood of those infected hosts (Garnham 1966). This movement of parasites through vertebrate and invertebrate hosts, establishes a circular flux of parasites running from human to mosquitos and from mosquitos to humans in endemic areas. Vertical transmission of malaria from infected pregnant woman to her foetus is also present in nature (Dominguez 1987, Meuris et al. 1993). Natural malaria transmission by other means are presently non accepted. The same mechanisms of transmission as described in human malaria are generally applied to malaria of other mammals, birds and reptiles (Garnham 1966). Previously we observed that malaria infection may be transmitted mouse to mouse by oral inoculation of blood from a mouse infected with *Plasmodium berghei yoelii* (Pby) to another clean mouse (Malagón et al. 1993).

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Taking into consideration that tissues contain vessels and vessels contain blood and blood contain the malaria parasites, we thought that malaria infection could well be transferred, also, through the ingestion of tissues of an infected animal.

To see if malaria infection take place when a clean mouse is fed on the tissues of another malaria infected mouse, that is to say, through cannibalism, we performed the experiments below described.

MATERIALS AND METHODS

The parasite used in these experiments was *Plasmodium berghei yoelii*, obtained from the London School of Hygiene and Tropical Medicine, in 1969, by courtesy of Professor PCC Garnham and Dr. D.E. Davidson, and maintained since then by mouse to mouse intraperitoneal passage. Male CD1 mice were used one week post-weaning, weighing from 14 to 17 g. In all experiments the number of experimental and control mice was the same. Six experiments were performed using in the whole 54 experimental and 54 control mice. Bait mice to be eaten by experimental mice were inoculated with infected blood by intraperitoneal route and four days later, before being sacrificed, blood films were prepared to check parasitemia.

Bait mice to be eaten by control mice were sacrificed at the right moment, without previously being infected. The thin blood films were giemsa stained and observed to assess the per cent parasitemia in 2000 erythrocytes. To start each experiment, control and experimental mice were isolated in a cage each, with no food but water permanently available, to enhance cannibalistic behaviour. After starvation of the mice all day long, the corpses of freshly killed infected bait mice were offered to experimental mice, late afternoon and for all night long, while the control mice were offered with clean mice. Next morning bait mice were inspected and a list of the non eaten organs was produced. After that, all mice reassume their normal diet. For detection of the malaria infection blood smears were prepared from all experimental and control mice, starting 5 days after baits were fed and twice a week thereafter until a month was completed. Thin blood films were giemsa stained and observed at 100X in light microscope. This experiment was repeated 6 times.

RESULTS

Parasitemia of all infected bait mice ranged from 10 to 78.9%, with a mean of 46.80%. All mice eat their baits. All mice eat muscle and no one eat intestine (Table 1). Mice showed individual preferences to eat other organs. Some of the mice fed with the corpses of infected bait mice acquired malaria infection, but no blood infection was possible to demonstrate in any mice fed with the corps of clean mice. From all experimental mice, 35% acquired malaria infection (Table 2). Infected mice showed an approximate prepatency of 1 to 3 weeks.

Table 1

Cannibalistic behaviour, ingestion preferences of experimental and control mice*

	M	B	H	Lu	Li	Sp	K	St	I
E & C	100	90	96	97	67	28	20	16	00
C	100	96	77	96	52	23	15	15	00
EI	100	100	94	100	68	32	21	10	00
ENI	100	77	91	94	65	20	8	17	00

* - in % , M - muscle, B - brain, H - heart, Lu - lung, Li - liver, Sp - spleen, K - kidney, St - stomach, I - intestine, E and C - experimental and control mice, C - control mice, EI - experimental infected mice, ENI - experimental non infected mice

Table 2

Malaria infection acquired by mice fed on corps of bait mice infected with *Plasmodium berghei yoelii*

Experiment	Mice number	Infected	%	Prepatency in weeks 1	2	3
1	10	1	10	1	0	0
2	6	4	66	1	0	3
3	10	1	10	1	0	0
4	10	2	20	0	2	0
5	10	8	80	8	0	0
6	8	3	37	1	2	0
Totals	54	19	$\overline{X}$ 35	12	4	3

However, 63% of them exhibited parasites during the first week, 21% during the second week and 15.7% during the third week. The proportion of infected mice varied from experiment to experiment, going from 10% in the lowest to 80% in the highest (Table 2). There seem to be no correlation between the tissues eaten by a mouse and the establishment of the malaria infection. Mice acquired the infection when fed on bait mice with parasitemia between 10% to 80%, and a mean of 39%. However, parasitemia of bait mice from the most successful experiment, ranged from 10 to 24%.

DISCUSSION

Natural transmission of the malaria parasites by other means different to the mosquito bite, and the transfer of parasites from infected pregnant host to her foetus, was lacking. In the experiments here described we demonstrated that malaria infection may be acquired by eating the flesh and organs of an infected animal of the same species. This phenomenon, not previously described, opens the possibility to include another mechanism for the malaria transmission in nature. The participation of an *Anopheles* mosquito in the transmis-

sion of mammalian malaria, as well as, other mosquitoes or *Lutzomyia* (Ayala, 1971) for malaria of other zoological groups, permits the parasite to spread among vertebrate hosts, taking out the parasite from its confinement of one host, and producing infective forms for a new one, almost always of the same species. In transmission by cannibalism the parasites present in the blood of a vertebrate host pass to the alimentary canal of a new vertebrate host of the same species, so transmission by cannibalism means that a *Plasmodium* species is transferred among vertebrate hosts of the same species, without the presence of a mosquito, and probably without the previous production of a special invasive form. We propose that this mode of transmission might be shared by other species of malaria parasites and hosts, as they occur under natural conditions. This kind of transmission would be restricted in nature to those vertebrate host species with natural cannibalistic behaviour, or be applied to any species forced to practice cannibalism due to food shortage pressure. Once demonstrated that malaria may be transmitted by ingestion of flesh and organs of an infected host, it seems to us that under certain circumstances the spread of malaria parasites would take place through alimentary chains in nature.

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ANNOUNCEMENT

SECOND EUROPEAN CONGRESS OF PROTISTOLOGY AND EIGHTH EUROPEAN CONFERENCE ON CILIATE BIOLOGY

The 2nd European Congress of Protistology and the 8th European Conference on Ciliate Biology will be held together in ClermontFerrand, France, from the 21 to 27 July 1995. This meeting will offer the possibility for european and oversea searchers working on the protists to meet, discuss and present their results on all aspects of the protistology (cellular and molecular biology, genetic, parasitology, ecology, systematics, evolution, etc...).

The scientific programme will be similar to that of the last International Protozoology Congress of Berlin (1993) comprising plenary lectures, symposia, contributed papers and posters sessions. Information about the scientific and organizing committees, accommodation, excursions and registration fees are in the first notice, now available.

For further information contact C. A. GROLIERE or G. BRUGEROLLE, Laboratoire de Biologie des Protistes, Universite Blaise Pascal de Clermont-Ferrand, 63177 AUBIERE Cedex, France; Tel. (33) 73 40 74 54 or (33) 73 40 7470; FAX (33) 73 40 76 70.

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Short communication

Contribution à l'Etude Biogéographique de *Centropyxis carinata* Chardez, 1964

Contribution of the Biogeographical Study from *Centropyxis carinata* Chardez, 1964

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Résumé. *C. carinata* observée en 1964 au Zaire, n'avait plus été revue, cette espèce à été retrouvée au Soudan. Ce travail permet de compléter son étude morphologique et écologique. Vivant dans les zones épiphytiques de diverses plantes aquatiques, elle semble avoir une répartition limitée au Continent africain.

Mots clés. Protozoaire, biogéographie.

Summary. *Centropyxis carinata*, observed in 1964 in Zaire has only been noticed again from aquatic samples from Soudan. These samples allow completion of its morphology and ecology. Living as epiphytic organisms on several aquatic plants, and settling limited to the African continent.

Key words. Protozoan, biogeography.

INTRODUCTION

Il est généralement admis, que les Protozoaires peuvent se développer dans toutes les stations possédant des conditions écologiques équivalantes, d'une façon mondiale.

En ce qui concerne les Thecamobiens, de nombreux Auteurs ont débattu de ces problèmes: Ehrenberg (1871) avait déjà constaté des différences faunistiques entre grandes régions, Van Oye (1944), Decloitre (1954), Cailleux (1978) ont abordé ces questions. Les travaux de Gauthier-Lièvre et Thomas (1958) semblent confirmer, en ce qui concerne les Difflugiidae, la présence de certaines espèces d'une façon endémique en Afrique.

Sagissant des espèces endogées, Bonnet (1983) admet que certaines espèces possèdent une distribution

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géographique restreinte, dont l'expansion reste tributaire du déplacement des biotopes par la dérive des Continents, donc de facteurs historiques.

Sans remettre en question les problèmes de cosmopolitisme et d'endémicité, il faut cependant reconnaitre que certaines espèces abondantes dans certaines régions géographiques, sont pratiquement absentes dans d'autres.

Il ne s'agit pas dans ces cas d'endémicité "sensu stricto" car le domaine de distribution se situe à l'echelle continentale.

En réalité, les espèces biogéographiquement intéressantes restent rares, il s'en trouvent cependant quelques-unes dans les Genres importants.

Les genres *Centropyxis* est important par l'abondance et la diversité des espèces dont les variétés sont fréquentes, les déterminations sont souvent un domaine riche en spéculations taxonomiques. Les efforts pour essayer de les éclaircir, sont le plus souvent basés sur des particularités mineures qui ne sont pas toujours héréditaires.

L'espèce dont il est question dans cette note possède une particularité morphologique unique dans le genre.

MATÉRIEL ET MÉTHODE

La première étude de cette espèce, portait sur des prélèvements provenant des Régions du Lac Bangwelo au Zaire (Leg.1962 Prof. Symoens).

Pour cette deuxième étude, je dispose du matériel provenant du Djebel Marra au Soudan (Leg.1965 Miss Mc Gowan), ces prélèvements fixés au formol neutre, m'ont permis d'étudier l'animal parfaitement conservé et de contrôler les variations intraspecifiques de la structure de la thèque.

Les espèces prélevées à la micropipette ont été isolées sur lames, certains spécimens ont été déshydratés et montés en préparations dans l'Euparal.

Les études cytologiques ont été faites après dilacération des thèques à l'aide de micro-aiguilles et coloration du cytoplasme et du noyau par le vert de méthyl acétique à 0,5 %.

DIAGNOSE

Thèque circulaire à face ventrale plane; pseudostome excentré circulaire ou ovale, s'invaginant plus ou moins fortement; sans brides internes.

La face dorsale est légèrement bombée et joint la face ventrale en formant une carène bien nette, toujours plus large à la partie postérieure de la thèque. Cette carène peut dans certains cas faire un tour complet de la thèque, mais le plus souvent elle prend naissance aux environs du tiers antérieur.

La thèque est jaune brunâtre assez foncée, sa structure est formée d'un vernis organique finement ponctué incrustant des particules minérales polymorphes. La carène apparait plus claire très souvent transparente, chez certains individus elle est presque exclusivement constituée par l'assemblage de Diatomées remaniées, alors que pour d'autres la majorité des individus du Soudan, elle est constituée de fins éléments minéraux et apparait plus pierreuse.

Dimensions: diamètre 180-215µm, épaisseur 50-55µm, pseudostome 60-70µm.

Le cytoplasme lorsqu'il est contracté, remplit environ les 2/3 du volume interne de la thèque, il apparait assez foncé, comportant de nombreuses inclusions représentant certainement des produits ingérés.

Le noyau est sphérique il mesure environ 35µm lorsqu'il est coloré. Un spécimen non coloré après compression entre lame et lamelle présentait un nucléole central et une membrane nucléaire bien détachée du suc nucléaire.

La coloration ne permet pas de distinguer les nucléoles.

ECOLOGIE ET REPARTITION

Région du Lac Bangweolo. Altitude 1140m (Zaire).

n° 9600. Groupement à Najas et Utriculaire, pH 6.4, 21.7°C.

n° 9613. Groupement à *Thalia welwitschii*, pH 6.0, 20.5°C.

Djebel Marra. Altitude 1700 m. (Soudan).

n° 2b Parmi les Algues filamenteuses sur le fond d'une rivière, pH 8.2, 17°C.

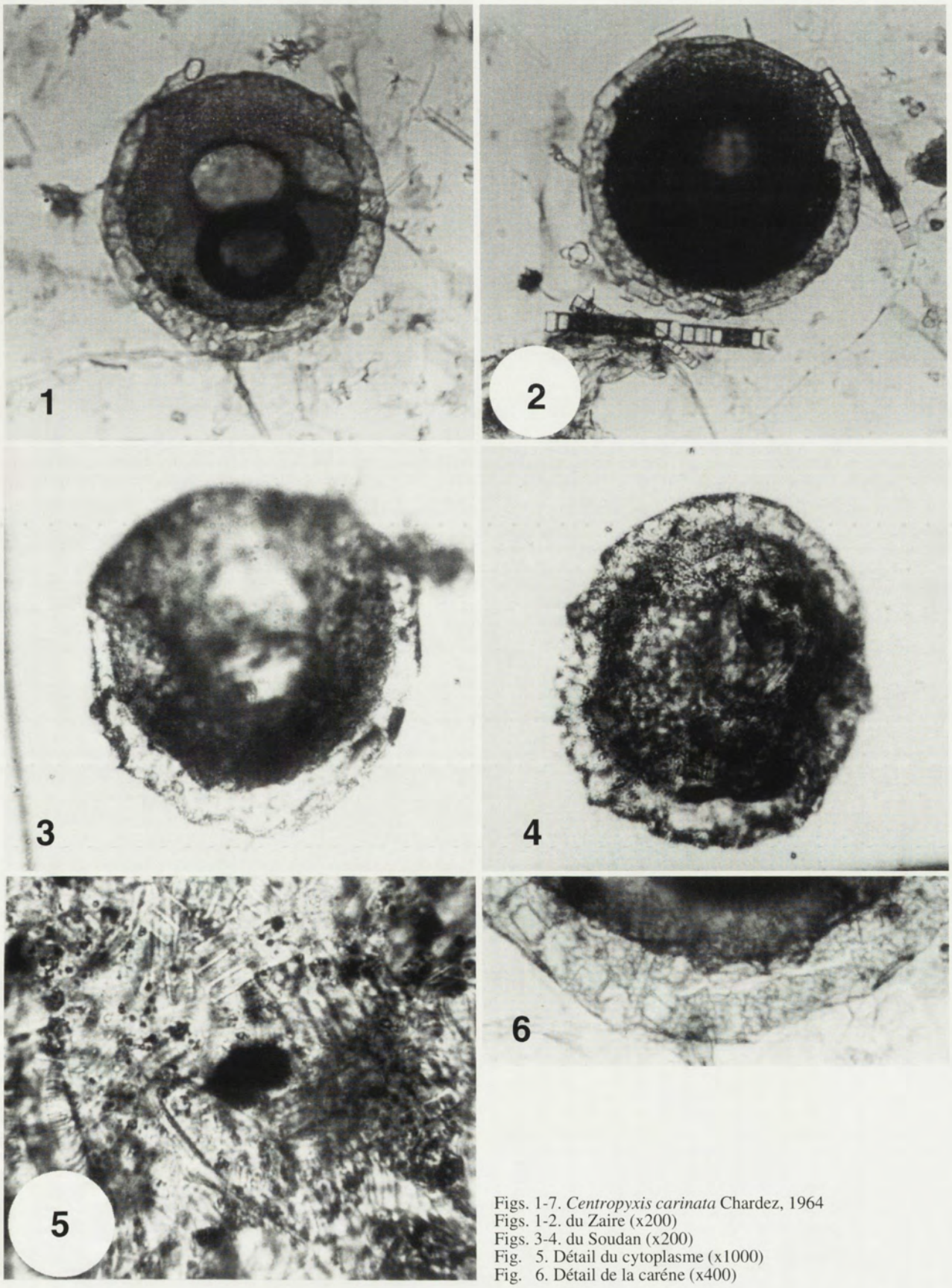
n° 3a Petite mare résiduelle, pH 8.2, 28°C.

Abondante dans ces échantillons, *C. carinata* paraît liée aux dépôts épiphytiques des plantes aquatiques.

Type: n° V2/5 Fac SC.Agr. Gembloux.

CONCLUSION

Centropyxis carinata est une grande espèce qui ne peut être confondue avec aucune autre, elle ne semble pas rare parmi divers végétaux aquatiques.



Figs. 1-7. *Centropyxis carinata* Chardez, 1964
Figs. 1-2. du Zaire (x200)
Figs. 3-4. du Soudan (x200)
Fig. 5. Détail du cytoplasme (x1000)
Fig. 6. Détail de la caréne (x400)

La diagnose que j'en ai donné en 1964 est avantageusement complétée par les spécimens provenant du Soudan.

Cette espèce est principalement caractérisée par la présence d'une carène plus ou moins large. La présence de cette carène augmente sensiblement la surface de la face ventrale du *Centropyxis* qui dans plusieurs cas a été observé fixé sur des débris végétaux.

L'étude du contenu du cytoplasme a révélé la présence de nombreuses inclusions de fragments végétaux mêlés à d'autres restes siliceux ainsi que des Diatomées de petite taille (*Achnanthe, Fragilaria*, et *Navicula*)

C. carinata semble bien être une espèce végétarienne, actuellement observée uniquement sur le Continent africain.

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Each table must be on a separate page. Figure legends must be in a single series at the end of the manuscript. References must be listed alphabetically, abbreviated according to the World List of Scientific Periodicals, 4th ed. (1963). Nomenclature of genera and species names must agree with the International Code of Zoological Nomenclature, third edition, London (1985) or International Code of Botanical Nomenclature, adopted by XIV International Botanical Congress, Berlin, 1987. SI units are preferred.

Examples for bibliographic arrangement of references:

Journals:

Häder D-P., Reinecke E. (1991) Phototactic and polarotactic responses of the photosynthetic flagellate, *Euglena gracilis*. *Acta Protozool.* **30:** 13-18

Books:

Wichterman R. (1986) The Biology of Paramecium. 2 ed. Plenum Press, New York

Articles from books:

Allen R. D. (1988) Cytology. In: Paramecium, (Ed. H.-D. Görtz). Springer-Verlag, Berlin, 4-40

Zeuthen E., Rasmussen L. (1972) Synchronized cell division in protozoa. In: Research in Protozoology, (Ed. T.T. Chen). Pergamon Press, Oxford, **4:** 9-145

Illustrations

All line drawings and photographs should be labelled with the first author's name written on the back. Figures should be numbered in the text as arabic numerals (e.g. Fig. 1). Illustrations must fit within either one column (86 x 231 mm) or the full width and length of the page (177 x 231 mm). Figures and legends should fit on the same page. Lettering will be inserted by the printers and should be indicated on a tracing-paper overlay or duplicate copy.

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Photographs at final size should be sharp, gloss finished, bromide prints. Photographs grouped as plates (in size not exceeding 177 x 231 mm including legend) must be trimmed at right angles accurately with edges touching and mounted on firm board. The engraver will then cut a fine line of separation between figures. Magnification should be indicated. There is a page charge for colour illustration.

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Authors will receive one set of page proofs for correction and are asked to return these to the Editor within 48-hours. Fifty reprints will be furnished free of charge. Orders for additional reprints must be submitted with the proofs.

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