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Ultrastructural Aspects of the Precystic and Cystic Cytoplasm of the Hypotrichous Ciliate, *Laurentiella acuminata* [1]

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Synopsis. During encystment, the cytoplasm of *Laurentiella acuminata* undergoes a series of changes at the cell organellae level. The resting cysts of this ciliate are included in the KR (kinetosome-reabsorbing cyst) group. A fusion of the four vegetative macronuclear masses takes place, during encystment, resulting in a single cystic macronuclear mass. There is a partial degeneration of the mitochondria. Precystic cytoplasm is very rich in rough endoplasmic reticulum. In precystic and cystic cytoplasm there are numerous autophagic vacuoles (autolysosomes), and the reserve substances of the resting cysts of *L. acuminata* are essentially lipidic inclusions and paraglycogen. The ultrastructural characteristics of the precystic and cystic cytoplasm of this hypotrichous ciliate are compared with the data found in other hypotrich ciliates of the *Oxytrichidae* family, showing characteristics in both precystic and cystic cytoplasm, which are common to those shown by other ciliates of the KR group.

A resting cyst is in a highly differentiated state, and its high degree of dehydration is the principal characteristic of this cryptobiotic state. Nutritional deficiency is the most effective and general factor in encystment, common to many protozoa. In this condition the organism has a behaviour similar to that of a "closed system" (Wright 1967), depending entirely upon endogenous materials for all the anabolic and catabolic reactions necessary for survival and morphogenesis. During encystment, precystic cells undergo a loss of cytoplasmic water and a decrease of cell volume. Simultaneously, there are changes at the cell

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organellae level, such as: the cortical ciliature, nuclei, mitochondria, ribosomes, vacuoles, lysosomes (autolysosomes), inclusion bodies and reserve substances. All these cell changes have a physiological signification that is not well known.

In this paper the ultrastructural characteristics of the precystic and cystic cytoplasm of the hypotrichous ciliate *Laurentiella acuminata* are presented and compared with the data found in other ciliates. The possible physiological significance in the described changes, their relevance and utility in Ciliatology are reported.

Materials and Methods

Organisms, Culture Conditions and Induction of Encystment

Laurentiella acuminata (Fedriani et al. 1976), a hypotrichous ciliate, was isolated from a sample of water collected in the "Parque de Maria Luisa" (Seville). *Chlorogonium* sp. was kindly given to us by Professor Ammermann of Tübingen University.

The cultures of *L. acuminata* were kept at $20 \pm 1°$ C in Pringsheim's medium and fed with *Chlorogonium* sp. The alga was grown autotrophically under white light (25 W/m²) at 30° C and aerated with CO_2 at 5% (v/v) in Pringsheim's medium.

In cultures of *L. acuminata*, encystment occurs when the number of food organisms diminish. The induction of encystment was carried out by placing the vegetative cells in a Pringsheim's solution without adding *Chlorogonium*.

Electron Microscopy and Cytochemical Procedures

Both precystic cells and resting cysts were fixed for an hour in a 1 : 1 mixture of solution-A (2.5% v/v glutaraldehyde in 0.05 M phosphate buffer at pH 7 with 1.0 mM $MgSO_4$ and 0.1 M sucrose) and solution-B (2% w/v OsO_4 in 0.5 M phosphate buffer at pH 7). The material fixed was embedded in 1.5% (w/v) agar, after dehydration the blocks were embedded in Epon resin.

Ultrathin sections were obtained with a Reichert-Jung ultramicrotome sections were, stained with uranyl acetate and lead citrate, examined with a Siemens Elmiscop-102 electron microscope.

For the demonstration of polysaccharides by electron microscopy, cells fixed only in solution-A were embedded in Epon, sectioned and mounted on a gold grids and stained according to the Thiery method (Thiery 1967).

Results

Cortical Ciliature

As previously determined, by optical microscopic observations (Gutierrez et al. 1981), the resting cysts of *L. acuminata* can be included in the KR (kinetosome-reabsorbing cyst) group (Walker and Mau-

g e l 1980). This implies that precystic cells reabsorb all the external cortical ciliature and infraciliature during encystment. Photographs (Pl. I 1–3) show three sucessive stages of the reabsorption of a cirrus. The first stage (Pl. I 1) is an early precystic state, the second photograph (Pl. I 2) shows the cirral reabsorption in a late precystic state, and in the third and last photograph (Pl. I 3) the cirral degeneration is observed in the later precystic state when the cyst wall is not still concluded.

Nuclei

During encystment of *L. acuminata* a fusion of the four vegetative macronuclear masses takes place (G u t i e r r e z et al. 1981), resulting in a single macronuclear mass. Possibly, this nuclear fusion produces a high condensation of the macronuclear chromatin, creating large condensed chromatinic bodies (Pl. I 4), in comparison to those observed in the vegetative macronuclear masses or precystic macronuclear masses (Pl. II 5). This may suggest that the chromatin, condensed like this, is in an inactive state.

During the fusion of the macronuclear masses, the "fusion bands", as they are called, have not been observed. Only a mixture of nuclear material and a fusion of nuclear membranes occurs (Pl. II 5).

Some micronuclei undergo reabsorption during encystment (G u t i e r r e z et al. 1981), but those that have not been reabsorbed are structurally similar to the vegetative micronuclei, and their chromatin does not undergo a condensation as high as that of the macronuclei. Sometimes, these micronuclei are covered with several amorphous and membranous layers.

Mitochondria

In *L. acuminata*, during encystment, there is a partial degeneration of the mitochondria (Pl. II 6), and the mitochondrial clusters are not formed. This does not result from a possible bad fixation because the mitochondria of the vegetative cells are not deformed or broken, then fixed by the same procedure. In mature resting cysts only a few scattered mitochondria and a large amount of mitochondrial residues are observed.

Ribosomes

Precystic cytoplasm is very rich in RER (rough endoplasmic reticulum) and scattered ribosomes, but a crystalline-like arrangement of ribosomes has not been found.

Autolysosomes

In precystic and cystic cytoplasm there are numerous autophagic vacuoles (autolysosomes), containing membranous material, numerous ribosomes and occasionally other cytoplasmic organellae such as mitochondria.

Inclusion Bodies and Reserve Substances

The reserve substances of the resting cysts of *L. acuminata* are essentially lipidic inclusions and paraglycogen. The paraglycogen granules usually appear in ellipsoidal form with a maximum diameter of about 0.66 μm and a minimum diameter of about 0.52 μm and there is a positive PATAg reaction (Pl. II 7).

In cystic cytoplasm, paracrystalline bodies are sometimes observed (Pl. II 8). Possibly they are of a protein nature, and their presence may be due to the high degree of cytoplasmic dehydration experienced during encystment. These bodies appear in both cystic cytoplasm and macronucleus.

Discussion

In Table 1 our observations on the characteristics of the precystic and cystic cytoplasm of *L. acuminata* are compared with the data found in the literature concerning other hypotrich ciliates of the *Oxytrichidae* family. All are resting cysts of the KR group (Walker and Maugel 1980); a common characteristic of all the ciliates of this family (Table 1). This is not so for all hypotrichs, since *Euplotes muscicola* (Faure-

Table 1

Comparison of the characteristics of the precystic and cystic cytoplasm in hypotrich ciliates

Ciliate*	A	B	C	D	E	F	G	References
Oxytricha fallax	+	+	+	+	+	+	+	Grimes (1973)
Stylonychia mytilus	+	+	+	+	?	?	+	Walker et al. (1975)
Pleurotricha sp.	+	−	+	+	+	+	+	Matsusaka (1976)
Gastrostyla steinii	+	+	+	+	+	+	?	Walker et al. (1980)
Histriculus muscorum	+	+	?	+	+	+	?	Matsusaka (1979) Matsusaka and Kimura (1981)
Laurentiella acuminata	+	+	−	+	+	+	+	This paper

* Hypotrich ciliates of the *Oxytrichidae* family. ? — no reported. A — KR group, B — macronuclear fusion, C — mitochondrial clusters, D — cytoplasm rich in RER, E — lipidic inclusions and/or paraglycogen, F — Autolysosomes, G — paracrystalline bodies.

Fremiet et al. 1954) and *Diophrys scutum* (Walker and Maugel 1980), both of the *Euplotidae* family, are in the NKR group.

Similary as in other eukaryotic cell differentiation processes, during encystment, the nucleus undergoes a drastic structural reorganization. In ciliates, these changes, may be experienced by both the macronucleus and the micronucleus, although the most impressive are those of the macronucleus. Many ciliates normally show various macronuclear masses in the vegetative state (Raikov 1982), and generally, during encystment these macronuclear masses fuse creating a single cystic macronuclear mass (Table 1). This involves a decrease of the macronuclear volume present in the vegetative cytoplasm, as in *Gastrostyla steinii* (Gutierrez and Perez-Silva 1981), in which a strong correlation between the loss of cytoplasmic volume during encystment (about 87.3%) and that undergone by the four vegetative macronuclear masses (~ 80%), is observed.

This fusion is accompanied by a high chromatin condensation probably resulting in the inactive state of the cystic macronuclear DNA. Possibly, the mutagenic effects of desiccation (inducer of encystment) on bacteria (Webb 1967) are reduced by chromatin condensation. The degree of chromatin condensation has been measured in the hypotrichous ciliate *Gastrostyla steinii* (Gutierrez and Perez-Silva 1981), by microspectrophotometry, using the C-Banding method. The results obtained indicate that cystic macronuclear chromatinic condensation is approximately twice as high as that of a single vegetative macronuclear mass.

At, the morphological level, these changes involve the appearance of large chromatinic bodies which are formed during encystment. What is generally observed is the transition from cord-like macronuclear chromatinic bodies (vegetative state) to spheroidal bodies. This has been observed in the following ciliates; *Oxytricha fallax* (Grimes 1973), *Stylonychia mytilus* (Walker et al. 1975), *Pleurotricha* sp. (Matsusaka 1976), *Gastrotyla steinii* (Walker et al. 1980), *Histriculus muscorum* (Matsusaka and Kimura 1981) and *Laurentiella acuminata*.

Macronuclear fusion also occurs in other cellular processes, such as cell division (Raikov 1982) or, as it is called, "paraconjugation" (Banchetti et al. 1980). In *Oxytricha fallax* (Grimes 1973), during macronuclear fusion, this author observed a "fusion band" structurally different from the macronuclear reorganization band, but it is structurally similar to those observed in *Oxytricha hymenostoma* (Banchetti et al. 1980) during paraconjugation. In *Laurentiella acuminata* this fusion band or a special region of fusion has not been observed.

There has only been fusion of the macronuclear membranes and a chromatinic mixture. In other hypotrich ciliates, such as *Diophrys scutum* (Walker and Maugel 1980), and rarely in *Pleurotricha* sp. (Matsusaka 1976) macronuclear fusion does not exist during encystment.

In both the macro- and micronuclear chromatin of some encysted ciliates, numerous microtubules have been observed (Walker et al. 1975, Walker and Maugel 1980, Walker et al. 1980, Matsusaka and Kimura 1981). Presumably they may play some role in chromatinic fusion and condensation.

Micronuclei do not undergo fusion, but degeneration of some micronuclei may occur during encystment, as has been observed in *Oxytricha fallax* (Grimes 1973), *Gastrostyla steinii* (Walker et al. 1980) and *Laurentiella acuminata* (Gutierrez et al. 1981). The micronucleus is sometimes covered with several membranous layers, as in *Stylonychia mytilus* (Walker et al. 1975), *Gastrostyla steinii* (Walker et al. 1980) and *Laurentiella acuminata*.

L. acuminata is the only hypotrichous ciliate of the *Oxytrichidae* family which does not show mitochondrial clusters (Table 1). On the other hand, a mitochondrial degeneration occurs during encystment, although, the cystic cytoplasm maintains some intact mitochondria. Also, in *Diophrys scutum* (*Euplotidae* family) there are no mitochondrial clusters.

It has been shown that a protein synthesis is involved in ciliate encystment (Matsusaka and Kimura 1981, Gutierrez et al. 1981). This protein synthesis provides evidence of an abundance of rough endoplasmic reticulum (RER). In *Oxytricha fallax* (Grimes 1973), *Pleutrotricha* sp. (Matsusaka 1976), *Histriculus* sp. (Matsusaka 1979) and *Laurentiella acuminata*, a cytoplasm very rich in ribosomes, which was in a polysomal state, was observed during encystment.

In *Oxytricha fallax* (Grimes 1973), *Stylonychia mytilus* (Walker et al. 1975) and *Pleurotricha* sp. (Matsusaka 1976), a crystalline-like arrangement of ribosomes was found, probably due a high cytoplasmic dehydration. This has not been observed in *L. acuminata*.

It has been reported that lysosomal enzyme activities are elevated during encystment. In *Histriculus* sp. (Matsusaka 1979) autophagic activities were detected from the earliest stages of encystment and the autolysosomes were abundant in their precystic cells. Also, in *Pleurotricha* sp. (Matsusaka 1976), *Oxytricha fallax* (Grimes 1973), *Gastrostyla steinii* (Walker et al. 1980) and *Laurentiella acuminata* (Table 1) autophagosomes were observed which contained membranous material, ribosomes, mitochondria and other cytoplasmic organellas.

In encysted ciliates as well as in resting cysts, inclusion bodies

and/or reserve substances have been observed, but their biochemical compositions are still not well known. In *Oxytricha fallax* (Grimes 1973) clusters of granules which appear to be similar to starch have been observed. In *Diophrys scutum* (Walker and Maugel 1980), clusters of glycogen-like material are evident in the early stages of encystment. This also has been reported in *Pleurotricha* sp. (Matsusaka 1976) and *Gastrostyla steinii* (Walker et al. 1980). In *L. acuminata* these inclusions are of paraglycogen in nature and are similar by the way the react to PATAg, size and morphology, to those described in other hypotrich ciliates of the *Oxytrichidae* family in the vegetative state (Verni and Rosati 1980).

Laurentiella acuminata, shows a series of characteristics in both precystic and cystic cytoplasm, which are common to those shown by other ciliates of the KR group. These characteristics and those reported in other papers (Gutierrez et al. 1981, Gutierrez et al. 1983) corroborate the inclusion of this ciliate in that group.

The study of the precystic and cystic characteristics of ciliates, may be good additional biological classification criterion for some groups of these protozoa.

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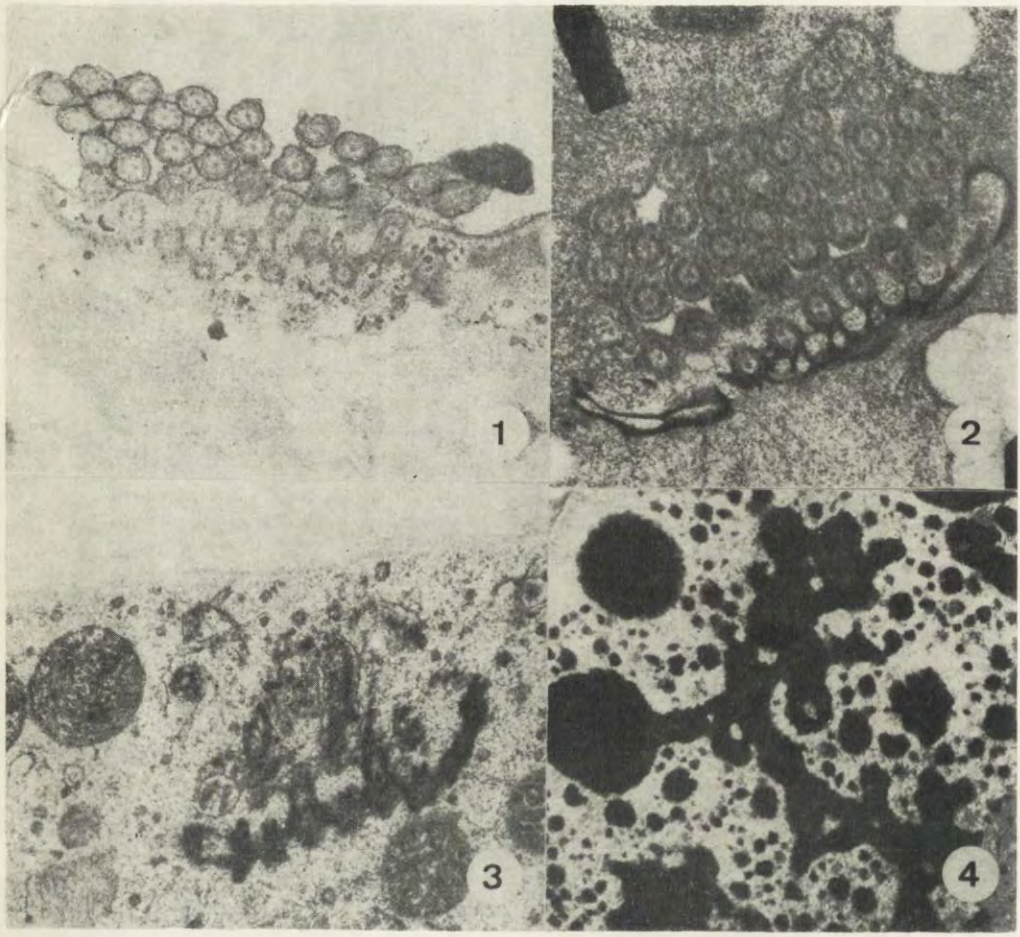
EXPLANATIONS OF PLATES I–II

1 to 3: Succesive stages of reabsorption during encystment, of the external cortical ciliature and infraciliature. 1 — Early precystic state. 25 000×, 2 — Late precystic state. 25 000×, 3 — Later precystic state. 25 000×
4: Large condensed macronuclear chromatinic bodies. 6000×
5: Mixture of nuclear material and fusion of nuclear membranes (arrows). 18 000×
6: Mitochondrial degeneration during encystment. 24 000×
7: Paraglycogen granules showing a strong reactivity to PATAg. 16 000×
8: Paracrystalline bodies 40 000×



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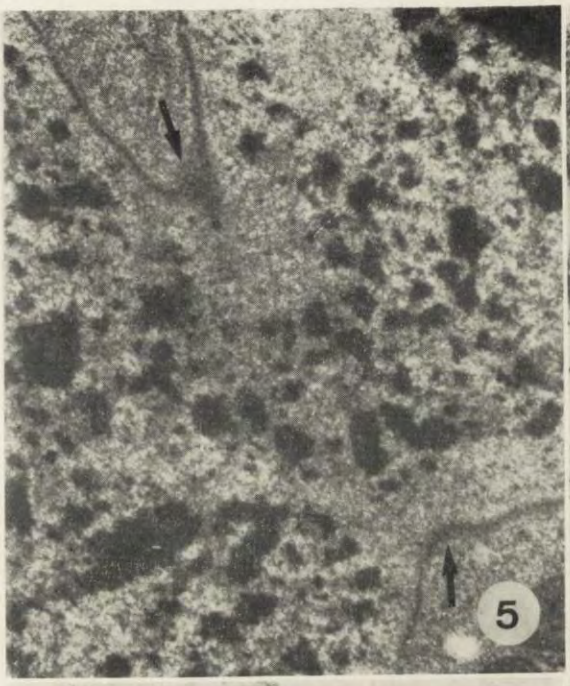

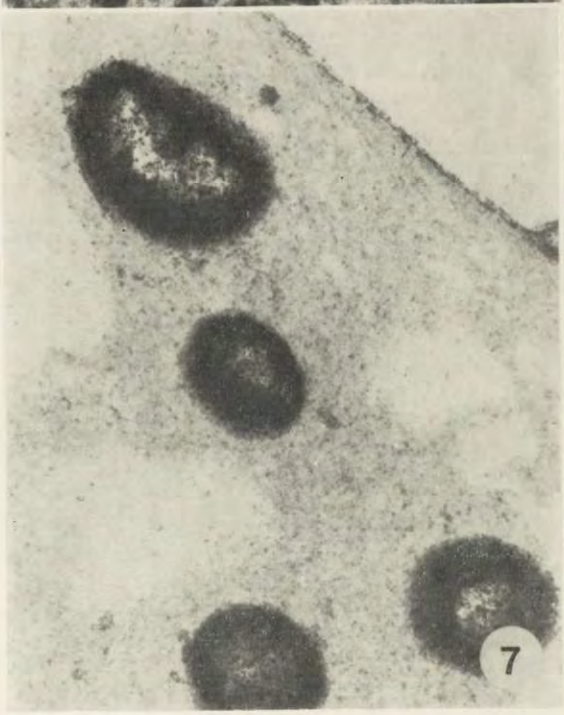

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The Question of Geotaxis in *Amoeba proteus*

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Synopsis. The method of steering amoeboid movement by dark stripes was used to study the geotactic response of *Amoeba proteus*. Velocities of locomotion of amoebae following the straight dark stripes were measured in the horizontal and vertical plane. The cells migrate in the vertical plane, independently of the direction of their motion: upward, downward or transversally, with approximately the same velocity as they move in the horizontal plane. Amoebae in the vertical plane were also obliged to follow dark stripes in the shape of Y or T and to choose the final direction along one of the branches. In the studied populations the slight majority of amoebae always chose the upper branch of the shaded path. It is concluded that *Amoeba proteus* manifests a statistically very weak tendency to the negative geotaxis.

The geotactic response of many species of *Protozoa* was studied in the quantitative way. The results have clearly shown that most free-swimming protozoans with undulipodia (Ciliates and Flagellates), such as *Paramecium* and *Euglena*, are negatively geotactic, i.e., they tend to swim upward in the absence of other stimuli and to accumulate at the top of cultures; see Bean (in press) for review. But there are also some positively geotactic species, such as *Blepharisma persicinum* (Fornshell 1980), which tend to swim down in the gravitation field and concentrate near the bottom of the container.

The influence of gravitation on the locomotion of *Amoeba proteus* has never been quantitatively studied. In the most extensive, and relatively recent review of the locomotory behaviour of amoebae (Bovee and Jahn 1973), all the information relating to geotaxis is limited to the following statements: "No analysis of response by amoebae to gravitational fields or magnetic fields has been presented in the literature.

It is common knowledge that amoebae usually tend to remain at the bottom of a container, even though they are capable of climbing vertically on a glass surface. No geotactic study of their movements has been attempted". Certainly it is the way amoebae migrate, which makes difficult any quantitative experiments. Amoebae locomote when they are attached to the substrate and in opposition to free-swimming protozoa they move very slowly. Their locomotion, characterized by extension of new pseudopodia in different directions and retraction of the old ones, makes each individual hardly comparable to the others. A possibility of controlling the cell migration and the cell shape by reduction of the fronts number and determination of the locomotion direction has been provided by employment of the stripes of shade (Grębecki 1980, Kłopocka and Grębecki 1982). Amoebae following the straight stripes of shade assume the orthotactic form with one single front. The individuals migrating along the dark stripes branching in the form of Y or T, after reaching the ramification form two fronts from which one becomes the tip of leading pseudopodium and the second is later obliged to retreat. Normally, without other stimuli, the choice of the direction of further locomotion is random.

This method of steering amoeboid movement by the dark tracks seemed to be appropriate to be used to study the geotactic response of amoebae in the quantitative way. The shades in the form of Y and T, when projected on the vertical plane, make the choice of movement direction unequivocal and uniform in each individual because amoebae following such stripes have the possibility of migration restricted only to these two directions: upward or downward. When amoebae are made to migrate along the straight paths of shade, on the horizontal plane or on the vertical one, it becomes possible to measure the velocity of their movement in the directions exactly determined in respect to the gravitation field.

Material and Methods

Amoebae were taken for experiments from the cultures of *Amoeba proteus* maintained in the Pringsheim medium and fed on *Tetrahymena pyriformis*. The well attached individuals were selected. They were transferred to the closed glass test-chamber filled with the same culture medium. A few seconds after the transfer amoebae attached to the bottom of the chamber and began to migrate. The chamber was put on the microscopic stage. The microscope was fixed to the stand which made it possible to change its position immediately from the vertical to the horizontal. Then obviously, the former bottom of the test-chamber with amoebae became its vertical back wall. The chamber was enough tight to allow such changes of position.

The straight dark stripes were cut from the Kodak gelatin neutral filter. The dark screens in the form of Y and T were prepared by photographic methods (Kłopocka and Grębecki 1982). Both branches of the Y formed the angle of 70°, which produces 35° deviation of new pseudopodia from the former direction of locomotion. That angle was demonstrated to be optimal as well in the free locomotion of *Amoeba proteus* (Sayers et al. 1979) as in the migration guided by the stripes of shade (Kłopocka and Grębecki 1982). But on the other hand it does not expose amoebae to the maximal possible effects of the gravitation field. On the contrary, the T-shaped shade which imposes 90° deviation to new pseudopodia is less physiological, but it utilizes in full extent the natural gravitation force. All types of dark screens were introduced into the optical path of the lighting system of the microscope. Their image (reduced about 10 times) was focused on the plane of cell migration using the substage condenser. The light intensity amounted to 8000 lux in the bright part of the field of view and was reduced approximately by the factor of 4 within the stripes of shade.

The microscope was equipped with the MPI-3 differential interference contrast device of Pluta System (PZO-Warsaw).

The experiments were run in a semi-dark room, at 18 ± 2° C.

Results

All the experiments were run under the conditions in which the choice of direction was perfectly random, when amoebae were moving in the horizontal plane. In the control tests they were made to migrate only horizontally along the dark stripes in the form of Y (Fig. 1 A), or T

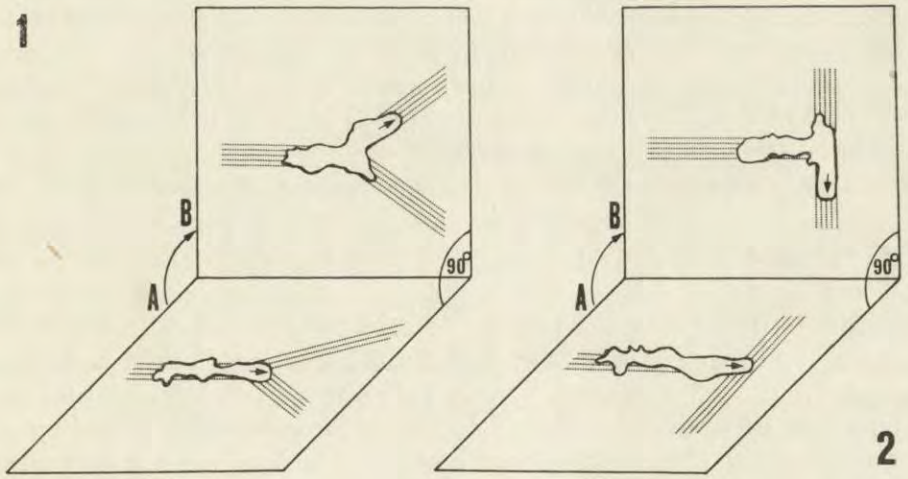

Fig. 1. Amoebae introduced into a stripe of shade in the shape of Y in the horizontal plane (A) make the choice of direction in the control tests in the same position, but are turned to vertical (B) in the geotactic experiments

Fig. 2. The same experimental situations as in Fig. 1, except the shape of the shaded paths which form a T

(Fig. 2 A). The final direction of locomotion was noted. In both cases exactly the same number of tested individuals chose the direction along each one of the two shaded branches. Such results show that there were no external stimuli which could influence the direction of movement. The observations of cell migration in the vertical plane were made under the identical conditions as the control tests, which guarantee therefore that no other external stimuli were influencing the cell locomotion except the force of gravitation.

Two series of experiments were run. In the first one the choice of the movement direction by amoebae migrating in the vertical plane was observed. In the second series, the velocities of locomotion along the dark stripes in horizontal and vertical position were measured.

In the first series of experiments amoebae migrating initially in the horizontal plane were introduced in the basal part of a dark stripe in the shape of Y or T. When they became orthotactic and their fronts approached the ramification point of two branches, the microscope with the test-chamber was turned by 90°. So, the consequent choice of further direction had to be made in vertical plane, under influence of the gravitation field (Figs 1 B and 2 B). Sometimes the whole procedure was started already in the vertical position. In each situation the final movement direction of 50 individuals was noted. When amoebae migrated along the shaded path in the form of Y, 30 individuals chose the way along the upper branch. For such distribution $\chi^2 = 2.00$, and the probability is between 0.20 and 0.10. So, there is a 80–90% chance that the difference between the number of amoebae migrating upward and downward really was significant. In the experimental situation in which amoebae followed the shade in the form of T, 29 individuals migrated upward and 21 down, what means there is slightly below 80% chance that the difference was significant.

In the second series of experiments amoebae were made at the beginning to migrate along a straight dark stripe in the horizontal plane (Figs 3 A and 4 A). When the position of the microscope was changed together with the test-chamber, they continued to move along the same stripe but in vertical position (Figs 3 B and 4 B). From that moment amoebae were guided by the dark stripes either upward or downward (Fig. 3), or transversally in respect to the gravitation field (Fig. 4). In each situation the velocities of locomotion of 25 individuals were measured (3 measurements per individual in horizontal position and the same number in the vertical plane). The average velocity of movement in horizontal position was compared with the average speed manifested by the same amoebae after turning them to the vertical plane (Table 1). The results prove that the average velocity of move-

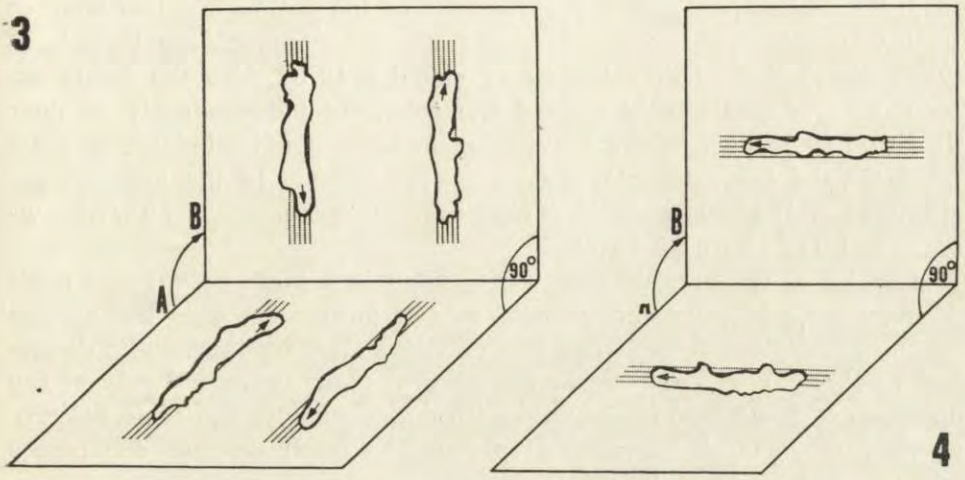

Fig. 3. Amoebae initially migrating along straight stripes of shade in the horizontal plane (A) are obliged to continue either strictly upward or downward after changing the position to vertical (B)

Fig. 4. Rectilinear migration started horizontally (A) and followed in vertical plane transversally to the gravitation force (B)

Table 1

Velocities of the rectilinear migration of *Amoeba proteus* in the horizontal plane, and then in the vertical one in three directions exactly determined in respect to the gravitation field

Position of the test-chamber	Velocity of movement (μm/s)		
Horizontal	2.85	2.90	2.89
Vertical	2.67 (upward)	2.76 (downward)	2.97 (transversally)

ment of the cells migrating in the vertical position does not depend on the upward, downward or transversal direction of their locomotion. The differences between the velocity of cells migrating in the horizontal and vertical planes are also statistically insignificant. In fact, they are often less pronounced than the differences within the population of studied amoebae due to their individual variation.

Discussion

Two informations may be found in the earlier literature which favor the view that the gravitation does not disturb the locomotion of amoebae. Under the conditions "immitating their natural environ-

ment" amoebae move along the threads of the cotton or glass wool as well when they are attached to the upper surface of the threads as when attached to their sides (Korohoda 1970). Also the freely extended pseudopodia bend toward the substrate independently of their direction in respect to the gravitation force. The extended pseudopodia bend toward the substrate when amoebae migrate in the inverted position as well as they do it during normal locomotion (Nowakowska and Grębecki 1978).

Results of the present research confirm in a large extent this point of view. In particular the velocity of cell locomotion does not depend on the gravitation force, what was clearly shown by the results described in this paper. Amoebae in the vertical plane, independently of the direction of their motion, migrate with approximately the same velocity as they move on the horizontal plane. The small velocity differences which were noted are not significant.

However, the gravitation force may influence in a certain way the direction of cell locomotion. In the two studied groups of amoebae migrating in the vertical position along the shades in the form of Y or T, a slight majority always chose the direction of locomotion along the upper branch of the shaded path. The distribution of the individuals migrating in two opposite directions was not exactly random. It might be considered as fulfilling the definition of geotaxis given by Bean (in press). According to it the term geotaxis is used "... for all behaviors that result in a non-random distribution or directional orientation of independent individuals, and are dependent on gravity and the active propulsive movement of the individuals". On this ground one can conclude that amoebae migrating in the vertical position tend to move upward, i.e., against the gravitation force.

It seems that such response may be correlated with the position of the cell gravity centre in respect to the site of cell attachment. Amoeba adheres to the substrate by the anterior part of its body (Bell and Jeon 1963, Haberey 1971, Opas 1978). According to Grębecki (1976), the most effective points of attachment are situated at the distance equal to 1/3 of the body length from the frontal extremity. So, the weight of the disengaged posterior body region situated behind the attachment area is higher than that of the frontal zone. As a result, in amoebae climbing on a vertical surface the uroid and the whole posterior body region is slightly sinking and sloping down. It may lead as well to a mechanical gradual declination of the locomotory axis as to the favorization of pseudopodia which were extended obliquely upward (because they remain under the directional influence of forces exerted by the endoplasmic streaming behind them — Kłopocka 1982).

It should be, however, stressed again that, although the vertical choice of direction by *Amoeba proteus* is not exactly random, its response to the gravitation force is far from being as regular and uniform as in free-swimming Ciliates and Flagellates. It is also much weaker and probably different in nature from other taxes manifested by amoeba, which are characterized by the choice of direction made already at the stage of formation of the new fronts, as under the influence of cathodal current (Mast 1931), chemical attractants (Korohoda 1977) and low illumination (Grębecki 1980). The negative geotoxis of *Amoeba proteus* represents only a weak statistical tendency, but not an unequivocal mass reaction.

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Electrical Properties of the Cell Membrane of a Marine Ciliate *Fabrea salina*

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Synopsis. Electrophysiological studies were performed on a marine ciliate *Fabrea salina*. Under laboratory conditions ciliates were exposed to solution: 1.1 M NaCl + 0.13 M $MgCl_2$ + 0.025 M $CaCl_2$ + 0.022 M KCl + 0.005 M Tris/HCl (pH 7.2). In this solution the membrane resting potential (RP) was −32 mV (± 4 S.D.). Depolarization of cell membrane was noticed when concentration of potassium ions in external medium was increased, while smaller depolarizing effect was observed in medium with high concentration of calcium. Removal of sodium from the medium was followed by membrane hyperpolarization up to −80 mV.

Intracellular constant current injections induced graded electrical responses of membrane in *Fabrea*, Voltage-current relationships were plotted for two experimental solutions: standard and standard "Na-free" (containing choline chloride for NaCl). The time of rise and the time of fall of action potentials (AP-s) evoked by intracellular injections of current were faster at higher levels of external calcium ions.

The appearance of graded membrane responses in Na-free solution and the dependence of AP shape on concentration of external calcium ions lead to conclusion that in *Fabrea* — similarly as in fresh water ciliates — calcium might be the cation carrying current throughout the cell membrane.

Addition of barium ions to external medium induced "all-or-none" AP-s, which showed longer duration when the ratio $[Ba^{2+}]_{ext}$: $[Ca^{2+}]_{ext}$ was raised.

Electrophysiological results on fresh water ciliates demonstrate that the excitability of the membrane is due to an inward regenerative current carried by calcium ions (Naitoh et al. 1972, Eckert 1972).

Existence of a slow inward current carried by Na^+ was discovered in the wild type and paranoiac mutant of the fresh water ciliate *Paramecium tetraurelia* (Saimi and Kung 1980). It has been recently reported for the marine ciliate *Paramecium calkinsi* that an electrogenesis of the membrane is calcium dependent (Deitmer and Machemer 1982).

The aim of the present study was to elucidate what ions are responsible for an inward, regenerative current throughout *Fabrea salina* cell membrane and how its excitability is influenced by the highly concentrated salt solution, in which this ciliate lives under normal conditions.

Material and Methods

Fabrea salina strain (obtained from the Laboratory of Zoology, Ecole Normale Superieure, Paris) was grown in medium:

1.1 M NaCl + 0.13 M $MgCl_2$ + 0.07 M Na_2SO_4 + 0.025 $CaCl_2$ + 0.022 M KCl + + 0.005 M $NaHCO_3$ + 0.005 M Tris/HCl (pH 7.2)

with addition of *Aerobacter aerogenes* as a standard food supply.

For the electrophysiological experiments standard control solution of following composition was used:

1.1 M NaCl + 0.13 M $MgCl_2$ + 0.025 M $CaCl_2$ + 0.022 M KCl + 0.005 M Tris/HCl (pH 7.2)

In all experimental solutions the ionic strength was kept constant. When a concentration of any component was increased (or when a new component was added) — the NaCl concentration was decreased by the osmotically equivalent amount.

The Na-free solution or solutions with lower concentration of sodium by comparison to control medium, contained choline chloride for NaCl. In the solutions with $BaCl_2$, the concentration of total amount of $BaCl_2$ and $CaCl_2$ was kept constant (held on the level 0.1 M) and an increase of $BaCl_2$ concentration was followed by a decrease of $CaCl_2$ concentration.

The electrical recordings were performed by means of two glass microelectrodes which were impaled into a single cell in a way described by Naitoh and Eckert (1968a). Microelectrodes were filled with 3 M KCl (20–40 MΩ). One microelectrode was used for recording and second one for intracellular injections of current. The membrane potential was measured as the voltage difference between intracellular microelectrode and extracellular reference electrode.

For measurements and recordings a standard set-up was used. The potential signal from the recording microelectrode was preamplified (Nihon/Kohden MZ-4) and recorded (oscilloscop camera facing the Tektronix 502A screen). To study the electrically evoked responses, the pulse generator (Medicor ST-3) was used

All experiments were performed at room temperature.

Results

Changes of Membrane Resting Potential in Various Ionic Composition of External Medium

The membrane resting potential (RP) in *Fabrea salina* measured in standard control medium was −32 mV (± 4 S.D.) and the resting input resistance in this solution was 20 MΩ (± 2.5 S.D.).

Under higher concentration of KCl in the medium the membrane was depolarized (curve "K" on Fig. 1).

The exchange of NaCl for choline chloride caused hyperpolarization of the membrane (curve "Na" on Fig. 1). In "Na-free" standard medium the RP value was −80 mV.

In higher concentrations of $CaCl_2$ the membrane was slightly depolarized, whereas decrease of calcium concentration was followed by a slight hyperpolarization of the *Fabrea* membrane (curve "Ca" on Fig. 1).

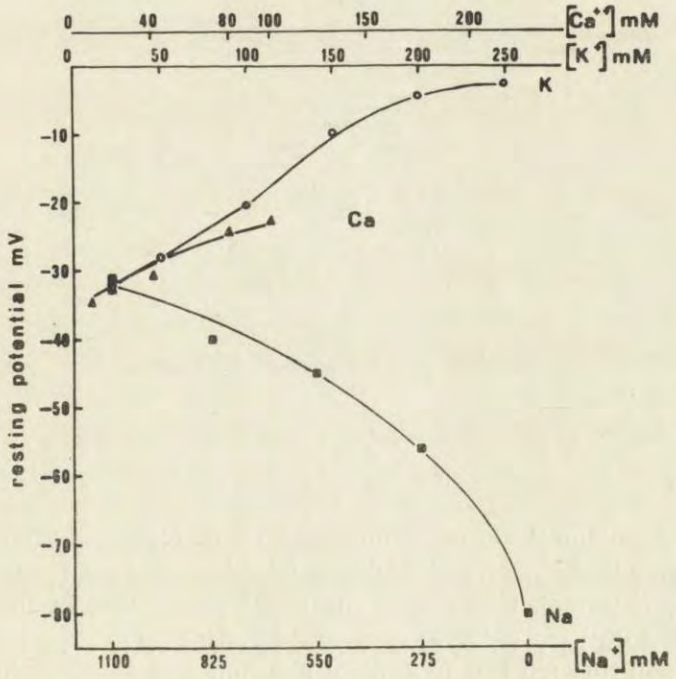

Fig. 1. Changes of RP in various ionic compositions of external medium: curve "K" — the effect of higher $[K^+]_{ext}$ on RP. Curve "Na" — the effect of removal of Na^+ from the medium on RP, curve "Ca" — the effect of various $[Ca^{2+}]_{ext}$ on RP (logarithmic scale).

Characteristics of Graded Action Potentials

Square, constant current pulses were intracellularly injected to *Fabrea salina*. Figure 2 shows graded responses of *Fabrea* membrane on three depolarizing and three hyperpolarizing constant current pulses (100 ms, 0.6–1.0 nA). Long lasting phases of repolarizations in all six graded membrane responses were stated.

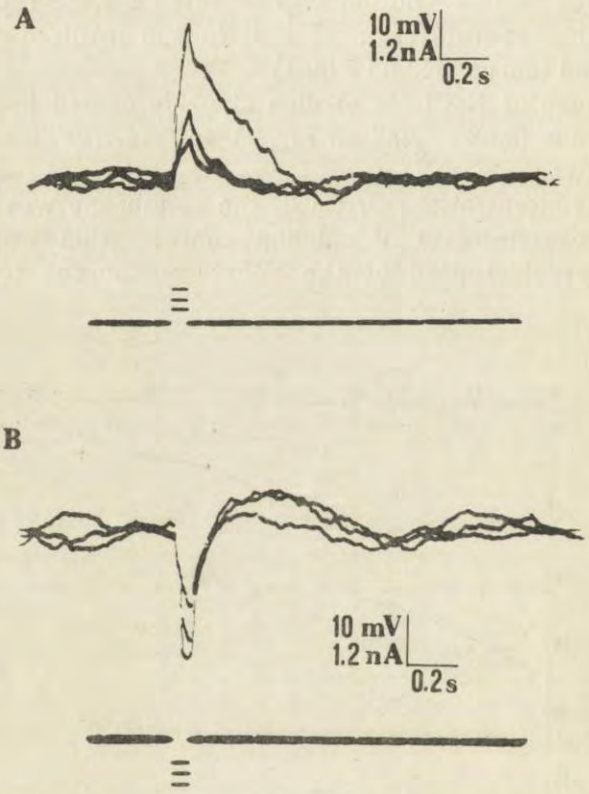

Fig. 2. Graded membrane responses of *Fabrea* on constant current injections: A — depolarizing, B — hyperpolarizing. Note the long phases of repolarization of AP-s

The I-V relationships were obtained from: (1) standard control medium, (2) standard "Na-free" solution, (NaCl in this case was exchanged for choline chloride). Comparing the input resistances in these two experimental solutions (Fig. 3) we could notice high increase of it in "Na-free" solution (20 MΩ in control medium and 47.5 MΩ in "Na-free" solution).

To see an effect of different Ca^{2+} concentration on action potential

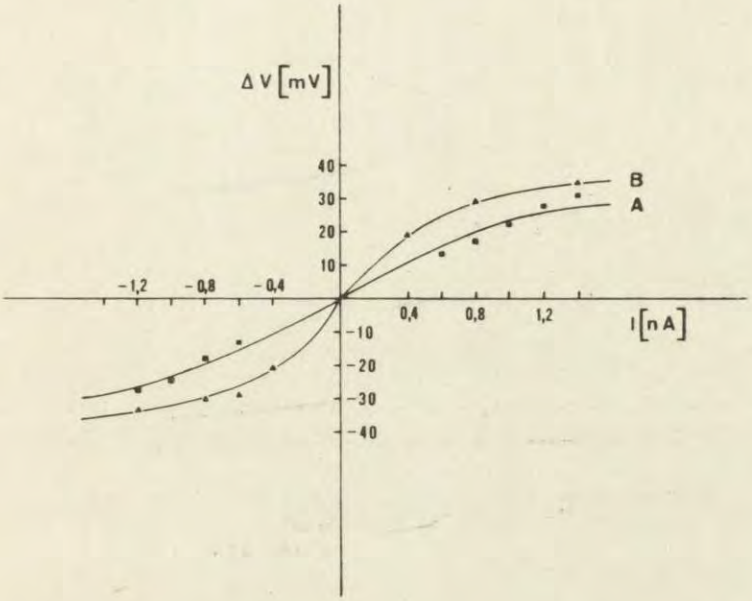

Fig. 3. I–V relationships plotted for: A — standard control medium, B — standard "Na-free" medium. Note the increase in input resistance in "Na-free" solution

generated by *Fabrea* after constant current pulse injection — one specimen was bathed in three experimental solutions of various Ca^{2+} concentration and stimulated in each of them. Middle trace on Fig. 4 was recorded in control medium (0.025 M $CaCl_2$), upper trace in the medium containing 0.1 M $CaCl_2$ and lower one in 0.004 M $CaCl_2$ (*Fabrea salina* can not survive in solutions where Ca^{2+} concentration is lower than 0.004 M).

The higher concentration of $CaCl_2$ the faster was the rate of rise of membrane response — in "low calcium" solution its time was 42 ms, in control — 21 ms and in solution enriched in Ca — 16 ms. Two phases could be recognized in the rate of fall of action potential in *Fabrea salina*. The first one — fast, lasting few miliseconds, started, when current pulse was terminated and this phase ended when the membrane potential value was nearly equal to the value of membrane resting potential. Since that moment the second phase — a slow one started. It lasted few hundred of miliseconds, while a value of membrane potential slowly attained the resting level. Both phases have become shorter, while the concentration of Ca was increased in the medium. The amplitude of all three responses was more or less the same.

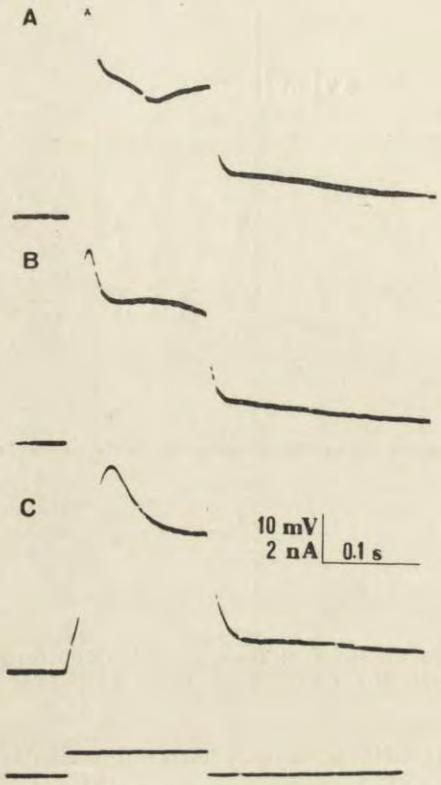

Fig. 4. Three membrane responses of one specimen bathed in solutions containing different concentrations of Ca^{2+} (A — 0.1 M $CaCl_2$, B — 0.025 M $CaCl_2$, C — 0.004 M $CaCl_2$). Note the effect of $[Ca^{2+}]_{ext}$ on the rise time and phase of fall of AP-s. See text for details

Characteristics of "All-or none" Action Potentials

As it was reported previously, *Fabrea salina* exposed to 0.01–0.02 M $BaCl_2$ (in presence of 0.016 M $CaCl_2$) showed frequently repeated short lasting ciliary reversal responses, so called "periodic ciliary reversal (PCR), which were associated with occurrence of "all-or none" action potentials (D r y l et al. 1982).

In the presence of 0.025 M $CaCl_2$ the threshold concentration of $BaCl_2$ for PCR induction was about 0.02 M. An increase of Ba^{2+} concentration caused prolongation of PCR response and also the time of renormalization of the cell motility was longer.

In experiments presented in this paper the total concentration of $BaCl_2$ and $CaCl_2$ was 0.1 M and was kept constant in all experimental

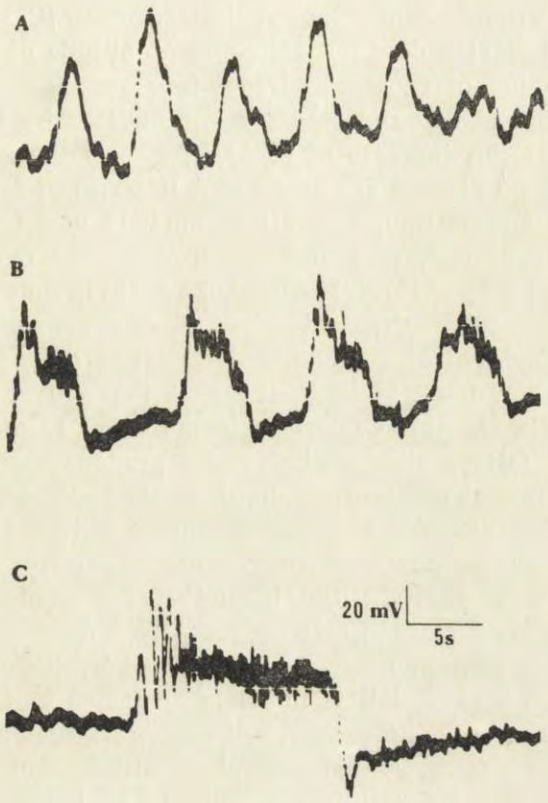

Fig. 5. "All-or-none" AP-s evoked by Ba^{2+} in solutions in which total amount of $BaCl_2$ was kept constant and the ratio $[Ba^{2+}]_{ext}$: $[Ca^{2+}]_{ext}$ was as follows A–1/3, B–1/1, C–3/1. Note the prolongation of AP-s in solution with the increased ratio $[Ba^{2+}]_{ext}$: $[Ca^{2+}]_{ext}$

solutions. A ratio of $[Ba^{2+}]_{ext}$: $[Ca^{2+}]_{ext}$ was changed in following way 1 : 3, 1 : 1, 3 : 1. Three traces recorded in those solutions are shown on Fig. 5. Worth to note is the prolongation of all-or-none AP-s as the ratio $[Ba^{2+}]_{ext}$: $[Ca^{2+}]_{ext}$ was raised.

Discussion

When taking into an experimentation a new object — as in this instance a marine ciliate *Fabrea salina* — one basic question has to be answered: to what extend the achieved results agree with the data achieved earlier on other ciliates?

The influence of various Ca^{2+} concentration on values of RP is the same as in case of fresh water ciliates. A membrane is depolarized as

$[Ca^{2+}]_{ext}$ is raised and slightly hyperpolarized when $[Ca^{2+}]_{ext}$ is lowered. An increase of $[Ca^{2+}]_{ext}$ does not influence amplitude of action potential evoked by injection of constant current into a cell — this result is at variance with the results reported for *Paramecium* by Naitoh et al. (1972) — whereas an increase of $[Ca^{2+}]_{ext}$ effects the rise time of AP, which is faster and steeper are also I and II phase of fall of AP. It has been found in *Paramecium,* that all parameters of an action potential (amplitude, time of rise, rate of repolarization and overshoot) depend on $[Ca^{2+}]_{ext}$ (Eckert 1972, Machemer 1976), but dramatic changes of amplitude were observed when $[Ca^{2+}]_{ext}$ was about 20 times lower than in the control medium. *Fabrea salina* did not survive when $[Ca^{2+}]_{ext}$ was below 0.004 M level, it means that only 6 times was possible to lower $[Ca^{2+}]_{ext}$ in comparison to the control Ca^{2+} concentration.

In phase of fall of electrically evoked graded responses of *Fabrea* membrane two phases can be recognized. Phase I — fast, which is referred to the voltage-dependent component of an outward current carried by K^+ and phase II — slow one, which could be referred to a Ca-activated component of K^+ current. $[Ca^{2+}]_{in}$-activated K conductance has been discovered in many metazoan cells — recently Adams (1982) reported that frog neurons contain K channels activated by $[Ca^{2+}]_{in}$. Taking into account that II phase of fall is much more faster in the test solution enriched in calcium (by the presence of external calcium the membrane is depolarized in comparison to control conditions and there is a possibility that calcium influx into the cell can be increased) it could be suggested that *Fabrea* also has a $[Ca^{2+}]_{in}$-activated K channels.

The great hyperpolarization of *Fabrea* membrane in "Na-free" solution (in "Na-free" RP = −80 mV, in control RP = −32 mV) and the dependence of evoked AP-s amplitude on $[Na^+]_{ext}$ indicate that external Na^+ ions strong effect the membrane excitability of *Fabrea*. The hyperpolarization of the membrane followed Na^+ removal may be caused by Na^+ efflux from inside of the cell to the outside which is a result of a new gradient of Na^+ concentration inside and outside of the cell. It is also possible that the change of gradient for $[Na^+]_{in}$ and $[Na^+]_{ext}$ may cause a change of g_{Ca} (such a mechanism was reported for squid axon (Baker et al. 1969, Blaustein 1974) and for barnacle muscle fibres (DiPolo 1973)). Comparing the I–V relationship plotted for "Na-free" and control medium we can see exactly that in "Na-free" solution the resting input resistance is higher. This result is in good agreement with data reported by Naitoh and Eckert (1968 a) from *Paramecium*. So, hyperpolarization of a membrane in "Na-free" solution may be the result of passive efflux of sodium ions from the inside of the cell and partly also because the membrane is less permeable for Ca^{2+}. Possible passive Na^+ efflux may also stimulate and change g_K.

Repolarization phase of "all-or-none" AP-s induced by Ba^{2+} ions is prolonged when the ratio $[Ba^{2+}]_{ext}$: $[Ca^{2+}]_{ext}$ is raised. The same results have been obtained for *Paramecium* (Naitoh and Eckert 1968 b) and *Stylonychia* (de Peyer 1973). Eckert et al. (1976) suggested that this effect is caused by a lack of activation by Ba^{2+} of the g_K component which is calcium dependent. Satow and Kung (1976) reported for *Paramecium aurelia* TEA insensitive mutant that Ba^{2+} blocks K channels and causes a long lasting repolarization. Blocking effect of Ba^{2+} on g_K in squid axon was discovered by Eaton and Brodwick (1980).

In conclusion, the presented results might indicate that excitability of a marine ciliate *Fabrea salina* is Ca-dependent and general electrical properties of its membrane are also very similar to those, which have been shown for fresh-water ciliates. As it could be expected, the great amount of NaCl in *Fabrea* external medium (1000 times higher concentration than in fresh water) effects its membrane excitability, although Na^+ ions seem not to be directly involved in generation of AP-s.

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The Inhibiting Effect of Ba^{2+} on K^+-induced Ciliary Reversal in a Marine Ciliate *Fabrea salina*

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Synopsis. Addition of Ba^{2+} to the high $[K^+]_{ext}$ medium, inducing everlasting ciliary reversal in *Fabrea salina*, inhibited the backward swimming. This effect could be observed only when concentration of external calcium was specified. Renormalization of ciliary movement was connected with repolarization of the membrane potential. It is suggested that inflow of Ca^{2+} in the presence of external Ba^{2+} may renormalize K-induced ciliary reversal in *Fabrea salina*, and calcium acts as Ca-channels inactivator and/or a factor which initiates an active removal of calcium from the cell into outside.

Under conditions depolarizing a cell mebrane the duration of ciliary reversal (CR) in *Paramecium* is constant providing that $[K^+]_{ext}:[Ca^{2+}]_{ext}^{1/2}$ is held constant (J a h n 1962). Square root relation between bivalent Ca and monovalent K suggests that ion competition in binding at anionic sites of a membrane plays an important role in the excitable properties of ciliary membrane. In other fresh-water ciliates (i.e., *Stylonychia mytilus*) the threshold concentration of K^+ for induction of CR response remains constant provided the $[K^+]_{ext}$: $[Ca^{2+}]_{ext}^{1/2}$ ratio is held constant (D r y l and de P e y e r 1970).

Fabrea salina is a marine ciliate which shows everlasting ciliary reversal while exposed to concentrations above 0.2 M KCl in the medium (D r y l et al. 1982), so the $[K^+]_{ext}$: $[Ca^{2+}]_{ext}^{1/2}$ ratio can not be a measure of a duration of CR response in itscase. Also the threshold concentration of KCl inducing CR does not depend on the above mentioned ratio.

The everlasting CR in *Fabrea* could be inhibited while adding Ba^{2+} ions to the medium without changing its ionic strength (K u b a l s k i 1981). The changes of polarity of ciliary movement in *Fabrea* associated

with the changes of its membrane resting potential under treatment with high K^+ and Ba^{2+} concentrations are described in this paper. The achieved results are used for explaining the mechanism of everlasting CR in *Fabrea* and for discussing general factors renormalizing ciliary activity.

Material and Methods

Fabrea salina strain (obtained from the Laboratory of Zoology, Ecole Normale Superieure, Paris) was grown in the medium:

1.1 M NaCl + 0.13 M $MgCl_2$ + 0.07 M Na_2SO_4 + 0.025 M $CaCl_2$ + 0.022 M KCl + + 0.005 M $NaHCO_3$ + 0.005 M Tris/HCl (pH 7.2) with addition of *Aerobacter aerogenes* as standard food supply. Cells used for experimentation were kept in unbacterized medium for 1–2 days. The standard control solution was:

1.1 M NaCl + 0.13 M $MgCl_2$ + 0.05 M $CaCl_2$ + 0.022 M KCl + 0.005 M Tris/HCl (pH 7.2).

The standard "low calcium" control solution contained 0.025 M $CaCl_2$. In all experimental solutions, the ionic strength was kept constant. When concentration of KCl was increased or when $BaCl_2$ was added — the NaCl concentration was decreased by the osmotically equivalent amount (for instance: in the test solution containing 0.3 M KCl + 0.240 M $BaCl_2$ — the concentration of NaCl was 0.342 M and concentrations of $CaCl_2$ and $MgCl_2$ were held on the levels as high as in the control standard medium). The movement of *Fabrea* was recorded using long-exposure dark-field photomacrographic method (Dryl 1958), time of exposition was 8 s.

Electrophysiological recordings and measurements of membrane potential were made in the way and on the set—up described elsewhere (Kubalski 1983). Membrane potential was measured first in control standard medium which was then replaced by perfusion with various test solutions. The last measurement of membrane potential was performed also in the standard control medium. The experimental chamber was perfused 4–5 times during an exchange of each test solution, by stimultaneous inflow and removal of bath solution. The differences of the reference potential level in various test solutions were 1–2 mV and they are not marked on Fig. 2, but the membrane potential values (given in Table 2) were determined according to the adequate reference level.

Results

The Effect of Ba^{2+} on CR Evoked by K^+

Ba^{2+} can inhibit CR while delivered to the medium containing high $[K^+]_{ext}$ in a sufficient amount. Table 1 shows concentrations of $BaCl_2$ necessary to be added to the test solution (without changing the ionic strength of the solution) for a recovery of a forward movement of *Fabrea salina*.

Table 1

Inhibiting effect of barium ions on potassium induced CR of *Fabrea salina* in dependence on concentration of external K

Concentration of KCl [M]	Threshold concentration of $BaCl_2$ inducing forward movement [M]
0.20	0.105±0.005
0.25	0.130±0.007
0.30	0.241±0.012
0.35	0.290±0.018
0.40	0.370±0.017

External solution contained in each case 0.05 M $CaCl_2$ + 0.13 M $MgCl_2$ + NaCl in amount required for keeping constant ionic strength of medium. Concentrations of $BaCl_2$ for recovery of a forward movement are expressed as arithmetical means from ten measurements.

The movement of the cells in test solution containing high concentration of KCl and $BaCl_2$ is much slower than in the control medium and no CR can be observed (Fig. 1 C).

In case when cells of *Fabrea salina* were exposed to mixed solutions of concentrations of KCl and $BaCl_2$ as high as mentioned in the Table 1, they did not show CR or very short lasting response if any.

The above mentioned amounts of $BaCl_2$ were not sufficient to abolish CR evoked by high $[K^+]_{ext}$ when the concentration of $CaCl_2$ in the medium was lowered to the level of 0.025 M.

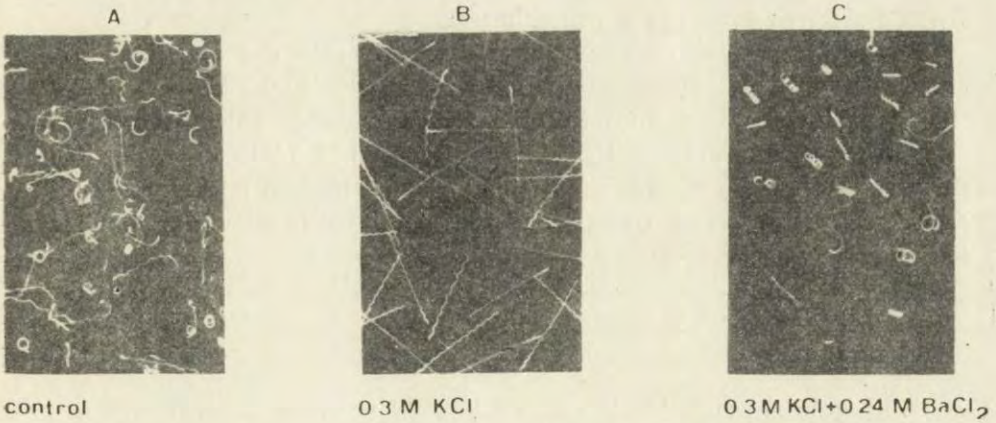

Fig. 1. Pattern of movement in *Fabrea salina* at various external conditions: A — forward movement in control medium, B — backward movement (CR) in high $[K^+]_{ext}$ medium, C — recovery of forward movement in high $[K^+]_{ext}$ medium after addition of $BaCl_2$

Changes of Membrane Resting Potential (RP) as a Result of Introducing Ba^{2+} into CR-inducing High $[K^{2+}]_{ext}$ Solution

The RP was measured in 6 test solutions: (1) control in presence of 0.05 M and 0.025 M $CaCl_2$, (2) standard 0.3 M KCl in presence of 0.05 M $CaCl_2$ and 0.025 M $CaCl_2$, (3) standard 0.3 M KCl + 0.24 M $BaCl_2$ in presence of 0.05 M and 0.025 M $CaCl_2$. Each cell was exposed to three test solutions (the level of $CaCl_2$ concentration was held constant in each of them) and RP was measured continuously. The results are shown in Table 2.

Table 2

Resting potential of *Fabrea salina* placed in external medium containing various concentrations of $CaCl_2$, KCl and $BaCl_2$

Medium	0.05 M $CaCl_2$*	0.025 M $CaCl_2$*
Control	-32.2 ± 3.8 mV	-31.4 ± 3.6 mV
0.3 M KCl	-2.4 ± 1.0 mV	-13.9 ± 2.1 mV
0.3 M KCl+0.24 M $BaCl_2$	-16.0 ± 1.8 mV	-15.0 ± 2.3 mV

* Concentrations of other cations as indicated in explanations for Table 1. Each value of RP is arithmetical mean from at least three measurements.

The RP value in the control medium was slightly lower when the solution was enriched with calcium. In 0.3 M KCl, the membrane was more depolarized in the presence of higher concentration of calcium and reached almost the same level in the both concentration of $CaCl_2$ when Ba^{2+} was added to the medium.

Figure 2 shows two traces recorded on two cells exposed to the same concentration of KCl (first to 0.3 M KCl and then to 0.3 M KCl + 0.24 M $BaCl_2$) while the test solutions differed as regards calcium concentration: trace A — 0.025 M $CaCl_2$, trace B — 0.05 M $CaCl_2$. Addition of $BaCl_2$ inhibited the K^+ induced ciliary reversal when $[Ca^{2+}]_{ext} = 0.05$ M and renormalization of a cell movement was associated with repolarization of membrane potential.

Discussion

A processs of inactivation of an inward calcium current and a mechanism of closing Ca-channels in the conditions of K^+-induced, long-lasting ciliary reversal are still not clear enough. K^+-induced CR can be reduced or abolished by extracellular application of ruthenium red

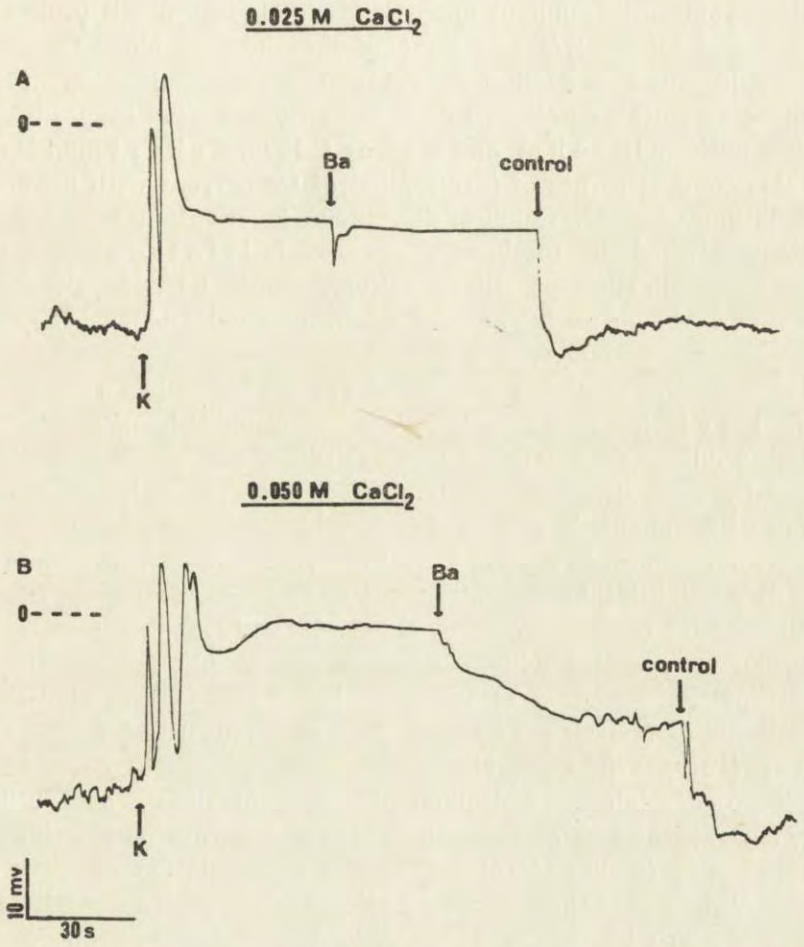

Fig. 2. Changes of membrane potential in *Fabrea salina* exposed to K and K/Ba factors at various concentrations of $CaCl_2$ in external medium. Note the change of RP after addition of Ba. Renormalization of ciliary movement occurred only in case "B". After exposition to the test K/Ba solution, cells were washed with control medium

(Onimaru 1976, Doughty 1978) which inhibits Ca^{2+}-ATPase. Application of verapamil or methoxy-verapamil (D-600) which are Ca^{2+} antagonists, effects in *Paramecium* complete or partial suppression of K^+-induced ciliary reversal (Doughty and Dryl 1981). On the other hand the CR response in *Paramecium* has a finite duration even if the stimulus inducing CR is maintained. Experiments performed on *Paramecium* showed that long exposition of cell to K^+ inhibited its excitability (Hildebrand and Dryl 1976, Dryl and Hildebrand 1979). These authors suggested, that during CR response cal-

cium ions coming throughout open Ca-channels into a cell progressively adsorb on the inner surface of a membrane and may block Ca-channels, what would cause a decline of CR and a renormalization of ciliary movement in spite of presence of K^+ ions which have induced CR.

Other authors (Brehm and Eckert 1978, Eckert and Brehm 1979) also connect a rate of inactivation of Ca-current with intracellular accumulation of Ca^{2+}. Calcium-dependent inactivation of Ca-channels was demonstrated in molluscan neurons (Tillotson and Horn 1978). Renormalization of ciliary activity would be a result of active Ca^{2+} transport from an intraciliary space by a voltage-sensitive calcium pump.

With reference to above mentioned proposal of Hildebrand and Dryl, Dryl (personal communication, unpublished) suggests that everlasting ciliary reversal in *Fabrea salina* at higher concentrations of K^+ may be a result of uncomplete effectiveness of calcium dependent blocking of Ca-channels from the inner side of a membrane. An additional factor responsible for appearing of everlasting CR in *Fabrea* could be the direct, inhibiting the activity of Ca^{2+}-ATPase effect of high concentration of K^+ (demonstrated by Doughty 1978).

Another mechanism which could be responsible for occurrence of everlasting CR in *Fabrea salina* could be perceived in a development of hypothesis of Machemer and Eckert (1975) and Eckert and Brehm (1979). When the ciliary mebrane is strongly depolarized in the presence of high extracellular cation concentration — an equilibrium between a rate of loss of intraciliary Ca and a rate of inactivation of the Ca-channel (which is related to the accumulation of Ca^{2+} inside cilium) could exist. When flows of calcium into and out of the intraciliary space are balanced, existence of constant Ca-current could be sufficient factor for maintainance of ciliary reversal.

Eckert and Brehm (1979) postulate presence of stabil potential difference that exists between inner and outer surface of the membrane, and its stability results from a potentiostatic feedback response of a membrane. In high ionic-strength medium, the extracellular cations neutralize negative surface charge of the membrane and thereby increase the potential difference between its outer and inner surfaces. In response, the membrane would exhibit a conductance change that favours entry of positive charge or loss of negative charge. If it is assumed that an active removal of calcium is voltage-sensitive, a sufficient amount of possitive charge must be introduced into the intraciliary space. When this condition is not respected the duration of excitation could be prolonged.

It is known that addition of Ba^{2+} into the medium highly activates

a transmembrane current ("all-or-none" potentials appear only in the presence of Ba^{2+} or Sr^{2+} (Kinosita et al. 1964, Naitoh and Eckert 1968). It can be assumed that the transmembrane current evoked by Ba^{2+} may introduce into a cilium a sufficient amount of calcium to inactivate Ca conductance and/or a sufficient positive charge necessary to restore the potential difference across the cell membrane and to initiate an active removal of calcium from a cilium into outside.

The results demonstrated in this paper may indicate that for recovery of forward movement in *Fabrea* rather inflow of Ca^{2+} than Ba^{2+} is required. Renormalization of a movement was observed when there was higher concentration of $CaCl_2$ in extracellular solution (0.05 M) and the $[Ba^{2+}]_{ext}/[Ca^{2+}]_{ext}$ ratio was lower i.e., 4.8 : 1. When the concentration of $CaCl_2$ in the medium was lower (0.025 M) and the ratio was higher i.e., 9.6 : 1, there was lack of renormalization. Ling and Kung (1980) demonstrated that influx of Ba^{2+} into the cell was much increased at higher values of the ionic ratio $[Ba^{2+}]_{ext}/[Ca^{2+}]_{ext}$ and is almost constant above the ratio 8 : 1. These authors showed also that Ba^{2+} influx depended on absolute values of $[Ba^{2+}]_{ext}$ and $[Ca^{2+}]_{ext}$ but only when the ratio $[Ba^{2+}]_{ext}/[Ca^{2+}]_{ext}$ was below 6 : 1, and was smaller while increasing $[Ca^{2+}]_{ext}$.

Summarizing — the transmembrane current carried by Ca^{2+} and Ba^{2+} may renormalize K^+-induced CR in *Fabrea* only when the extracellular conditions allow for entry of sufficient amount of calcium into the cell. It is still not clear, however, in what way Ca^{2+} inflow acts — as Ca-channels inactivator and/or a factor initiating an active removal of calcium into outside.

To the above mentioned factors which may influence a renormalization of ciliary activity in the presence of high $[K^+]_{ext}$, we must add a possible lack of an outflow of potassium ions from an intraciliary space, because of high level of these ions in the medium.

When KCl and $BaCl_2$ were simultaneously introduced to the medium in amounts required for renormalization *Fabrea salina* did not show CR and in the presence of Ba^{2+} the threshold concentration of KCl for induction of CR was higher. It can result from the immediate balance of Ba^{2+} or/and Ca^{2+} the potential difference across the membrane which is caused by depolarizing effect of K^+.

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Interrelationship Between Endocytosis and Cell Cycle of Histamine-stimulated *Tetrahymena*

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Synopsis. Tetrahymena pyriformis (strain GL) exposed to external medium enriched with histamine (10^{-6}M) showed increased rate of phagocytosis both in histamine pretreated and not pretreated populations. Nevertheless, the presence of histamine in external environment did not influence the interstage variations of phagocytotic activity observed during cell life cycle.

Among different types of endocytotic processes in the cell, the phagocytosis presents a convenient test commonly used in physiological studies of protozoa. It is known from earlier reports that phagocytotic activity in *Tetrahymena pyriformis* changes greatly in relation to the stage of cell life cycle. It is known that the process of endocytosis is completely inhibited during short period of cell division, but is present, in all other stages of cell cycle. Endocytosis can be evoked by various factors influencing the uptake of suspended particles. The phagocytotic activity in ciliated protozoa depends particularly on the nature of suspended material, which may be of natural (bacteria, yeasts, algae etc.) or artificial origin (like: carmin, chinese ink, coal or polystyrene particles). All these factors are able to induce the process of phagocytosis, which can be additionally enhanced or inhibited by other substances present in the medium. Histamine, for example, evokes the acceleration of phagocytosis in mammalian cells (Jancsò 1955, Kushinsky et al. 1951) and this effect was also found in the cells of lower vertebrates

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(Csaba et al. 1975) and in unicellular organisms, among them in *Tetrahymena pyriformis* (Csaba and Lantos 1973, 1977, Csaba et al. 1982).

Since *Tetrahymena* seems to represent a good model for observations of phagocytotic activity, it appeared interesting to use this unicellular organism in experiments which would elucidate the possible modifying effect of histamine (as well known stimulating agent of endocytosis) on the process of phagocytosis during various stages of the life cycle.

Material and Methods

The cultures of *Tetrahymena pyriformis* (strain GL) were cultivated on 1‰ yeast extract containing 1% Bacto trypton solution (both substances Difco, Michigan, USA), at temperature of 28°C. Two days after inocculation two heat shocks were applied according to method described by Zeuthen (1971). Synchronized cells were afterwards exposed to Chinese ink suspension in 4 ml of Losina-Losinsky medium (pH 5.7) in 0 min and subsequently at ten min intervals up to 110 min since cell cycle synchronization. This procedure was used paralelly for *Tetrahymena* cultures both pretreated with histamine solution (10^{-6}M histaminum hydrochloricum, Reanal, Budapest) 10 min before addition of Chinese ink and for control ones, not exposed to this chemical. The animals could form the f.v. with particles of Chinese ink for 10 min, afterwards were fixed with 4% formaline, washed with phosphate buffered saline (pH 7, 2), spread on microscopic slide and dried. The number of formed f.v. was established in samples containing 50 ciliates, both in control and experimental series. Experiments were repeated three times and the mean number of f.v. per animal was calculated. Student's test was used for the final analysis of achieved results.

Results and Discussion

The results of experiments are presented on Fig. 1. It was found that the immediate postsynchronization level of phagocytotic activity in control cultures of *Tetrahymena pyriformis* expressed as a mean number of food vacuoles formed during 10 min of exposure to Chinese ink particles was greatly decreased in comparison with that observed after 40–50 min of exposure. The highest number of food vacuoles appeared 50 min after synchronization (ca 8 f.v. per cell). The number of f.v. was decreased again (to approx 5 f.v. per cell) and then was growing again to nearly 7 after 70 min, with tendency to decrease during next stages of the cell cycle.

Thus, *Tetrahymena* cells showed maximal phagocytotic activity during synthetic stage of the cell cycle which is related to period of approxi-

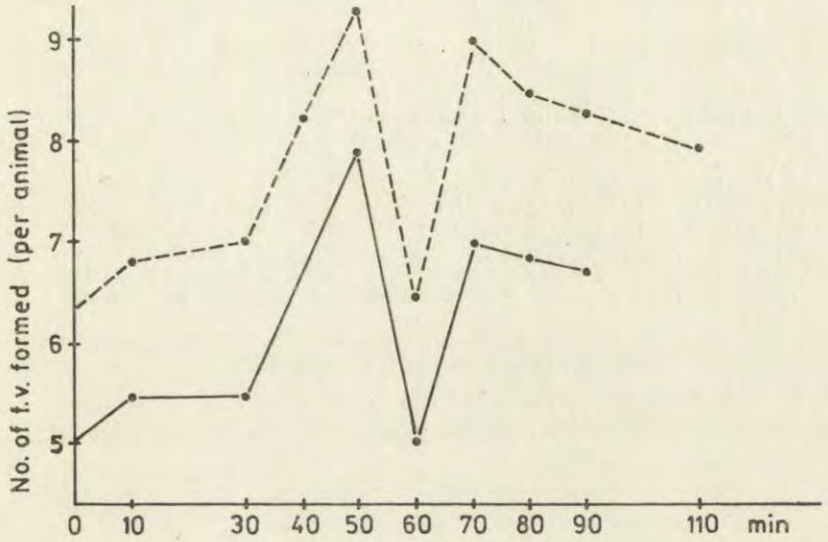

Fig. 1. Post cell-synchronization phagocytotic activity of control (solid line) and histamine-treated (dashed line) *Tetrahymena*. Each point of the diagram indicates the average number (mean of the three experiments) of formed food vacuole per animal during 10 min long intervals. It was proved by Student's test that differences between corresponding points of two curves were significant ($P < 0.5$)

mately 50–60 min after the end of thermic shock. The consecutive decrease and increase of phagocytotic activity is connected with the next cell cycle. The shape of the curves of the cyclic phagocytotic activity similar in all series of experiments be regarded as a general feature and agrees well with the earlier observations of Chapman-Andresen and Nilsson (1968), Nachtwey and Dickinson (1967), Nilsson (1976), and Rasmussen (1974).

The similar fluctuations in the course of phagocytotic processes found in histamine-stimulated cultures confirm the earlier findings of Csaba and Lantos (1973) but at a significantly higher level of values. This indicates that the degree of endocytosis in *Tetrahymena pyriformis* is controlled by the cell cycle.

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New Contributions on Parasitic Coccidia of the Hepatic Region of *Glossobalanus minutus* Kow. (*Enteropneusta, Hemichordata*)

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Synopsis. A light microscope description is made of various schizogonic stages observed for the first time in the epithelium of the hepatic region of *Glossobalanus minutus* Kow., with characteristics different from those of *Eimeria beauchampi* Leger and Duboscq, which are also described in this work. In addition, the extra-epithelial propagation of these parasites is demonstrated, on observing a large number of merozoites in the lumen of hepatic sacculations. Three types are differentiated according to their morphological and cytological characteristics.

In spite of there being a large bibliography on Coccidia, very few studies exist on these parasites in Enteropneusts.

Spengel (1893) observed a range of forms belonging to Sporozoans in the ciliated groove of *Glossobalanus sarniensis* Köehler, and in the esophagus of *Glandiceps hacksi* Marion, the nature of which, coccidian or gregarine, he did not determine.

Leger and Duboscq (1917) found various phases of schizogony and gametogony of a coccidium which they named *Eimeria beauchampi,* in the hepatic caeca of *Glossobalanus minutus* Kow. Furthermore, in the proboscis of the Enteropneust, they observed forms of schizogony very different from that described for the hepatic caeca, creating the species *Eimeria epidermica* of which no other information is known beyond that contributed by those authors.

More recently, a study of *E. beauchampi* has been published on macro- and microgamonts, which included histochemical results (Benito 1977).

In the present study the schizogony of *Eimeria beauchampi* observed in the hepatic region of *Glossobalanus minutus* is described and is designated as schizogony A. Also described are schizogonic forms (schizogony A) located in the same region and not observed previously by other authors. The possibility is suggested that these constitute a new generation of schizonts of *E. beauchampi* or that, in view of their characteristics, elements belonging to the schizogony described by Leger and Duboscq for *E. epidermica*, are involved.

Finally, for the first time, the presence of a large number of infectious germs in the lumen of hepatic sacculations is commented.

Materials and Methods

For this study specimens of *Glossobalanus minutus* Kow. have been used which were obtained from the beach of Luanco, Asturias. The specimens were kept several hours in sea water so that the sand contained in their digestive tract could be evacuated, they were then fixed using: Bouin, Zenker and 10% formaline in sea water fixatives.

Inclusion was made in paraffin and the blocks were cut to 5 and 7 μm in thickness. The following staining methods were used after the customary processes of paraffin removal and hydration: Heidenhain's azocarmine, Masson's triple stain, Mallory's triple stain and Heidenhain's iron hematoxylin. In addition, the following histochemical techniques were used: Alcian blue (AB), metachromasia with Toluidine blue (TB), Periodic acid Schiff (PAS) according to Hotchkiss and McManus, Tetrazoreaction of Danielli, Ninhydrin-Schiff (Yasuma and Itchikawa), and Dinitrofluorobenzene (DNFB).

Results

Not only schizogonic processes but also the numerous infectious germs observed were located in the hepatic diverticula of *Glossobalanus minutus* Kow. Fernández and Benito (in press).

Schizogony A: consists of very small elements (Fig. 1, Pl. I 2–5). The youngest, 2–3 μm, showed spherical forms and a small nucleus. On growing, the trophozoite kept its form. Between 5 and 6 μm in diameter, it showed a clear nucleolus, intensely stained by hematoxylin. The cytoplasm, which had a uniform or slightly granular aspect, coloured blue or red with Heidenhain's azocarmine, reacting positively to PAS, as well as to ninhydrin-Schiff, DNFB and tetrazoreaction.

Young schizonts were distributed in small groups, either in columns or parallel to the surface of epithelium, on the layer of nuclei. Among them, or located nearby, there were bi- or multinucleated schizonts

with an average size of 7 μm. Mature stages, located at different points of the epithelium, contained 2 to 12 small merozoites (between 4 and 6 μm in size), generally in a keg or "barrel" distribution. The merozoites showed pointed ends with central or posterior nuclei.

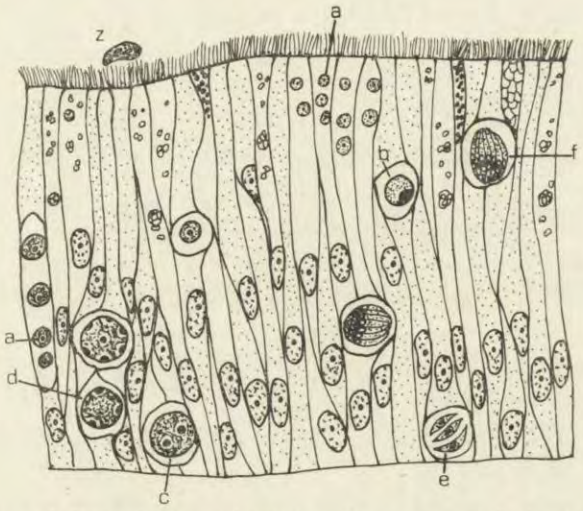

Fig. 1. Schizogony of *Eimeria beauchampi*: a–b — youngest stages, c — binucleate schizont, d — plurinucleate schizont, e–f — schizonts containing mature merozoites, z — free merozoite

This type of schizogony was observed in two specimens. In one of them, the schizonts formed compact groups at different points of the epithelium in the rearmost folds of the hepatic region and the terminal forms contained a varying number of merozoites. Macro- and microgamonts could be seen in the front folds, with an average size of 17 and 10 μm, respectively.

In the second specimen, the schizonts occupied the first sacculations of the middle of hepatic zone. The adult forms or "barrels" (with 8 to 12 merozoites) showed a superficial location, with an occasional domed epithelium being observed, about to eject the merozoites.

Schizogony B: The youngest forms were found here, from the mid-point of epithelium to its base, arranged in columns and with the smaller ones near the basal membrane. All had a spherical form and their diameter varied from 2 to 5 μm. The smallest showed indistinct nuclei, while the larger ones had a clear, sometimes excentric nucleolus.

In its development, the parasite becomes longer and acquires an oval shape. In this phase, its average size was 7 × 3 μm (some of the structures observed measured: 6 × 3 μm; 7 × 2.5 μm; 8 × 2.5 μm and 7 × 5 μm). Generally they were found in small groups and located parallel to the surface, in the middle of the epithelium. Their cytoplasm

contained very small granules and their nucleus, about 2 μm, showed a nucleolus which was always visible, of 1 μm (Fig. 2 A, Pl. II 6).

In a more advanced stage of development, the immature schizonts were found near the surface of the epithelium, sometimes in numerous groups (up to 16). The majority kept their oval shape (although some had a pear shape), reaching 23 μm in length. (Fig. 2 B; Pl. II 7). Most

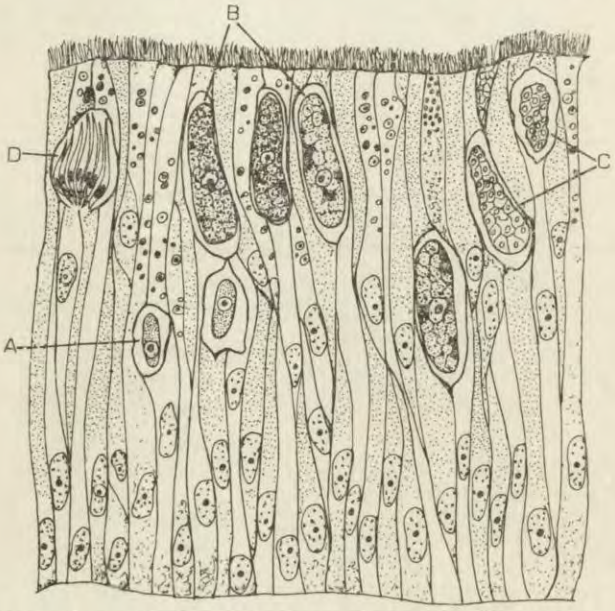

Fig. 2. Schizogonic stages in the anterior hepatic epithelium. A — early schizont, B — larger schizont with single nucleus, C — immature schizont with many nuclei, D — mature schizont

of these structures showed cytoplasm with small, sometimes very refracting, granules. Others, however, showed cytoplasm with a vacuolated aspect. In any event, the cytoplasm was stained fuchsia with Masson's triple stain, and brownish with hematoxylin, reacting positively to PAS.

Some large granules or inclusions could be seen in the cytoplasm and were usually distributed in the periphery of the parasite or near the nucleus. These granules were stained with hematoxylin, and were very orthochromatic with toluidine blue, reacting positively to tetrazo-reaction.

The nucleus, generally located in the center of the parasite, measured between 2 and 3 μm in diameter, with a nucleolus of 0.5 to 1 μm. Occasionally two nucleoli could be seen as well as chromatin granules.

In between these structures or near them, there were bi- or multinucleate forms with generally oval or pear shapes. Most common were those showing between 8 and 16 nuclei, with sizes varying between 8 and 20 μm × 5–6 μm (Fig. 2 C).

The nuclei had a clear and uniform nucleoplasm, with a distinct nuclear membrane stained blue with azocarmine and with Mallory's triple stain, reacting positively to ninhydrin-Schiff. The nucleoli, generally spherical, acquired reddish hues with azocarmine and black tones with ninhydrin. Only their perimeter generally took on color (Pl. II 8, 9).

Near these structures small groups (from 2 to 6) of adult schizonts could be seen, located on the surface of epithelium. The merozoites (10 to 11 μm in length and 1 μm in width) had a "barrel" distribution. The nucleus of each merozoite was located slightly posterior to the middle of the parasite and occupied nearly the width of the body (Fig. 2 D, Pl. II 10).

Infectious germs: These were observed in three of the specimens studied. In one of them, the infestation was so plentiful that the whole epithelial edge of the front hepatic sacculations was covered with them (Pl. III 11, 12). Many of them could be seen to penetrate the epithelium while others were inside it.

According to their morphological and cytological characteristics, three types have been differentiated:

(1) The most numerous and the largest type. The posterior end may be round or slightly pointed. The body of the zoites enlarged at the middle and then narrowed down again in a sort of elongation. Towards

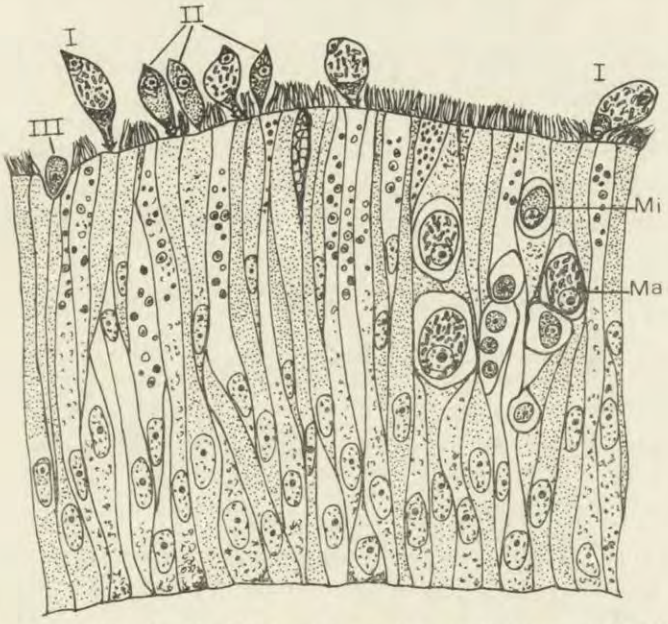

Fig 3. Free merozoites (I, II, III types) on the epithelium surface, and macro- (MA) and microgamonts (Mi) in the epithelium

its end, a spherical corpuscule of 1 μm, as well as an arrow-shaped termination could be seen (Fig. 3, Pl. IV 13).

The cytoplasm contained colorless and refracting, generally lengthy granules and among them small ones that were stained red with Heidenhain's azocarmine and Mallory's triple stain. The front elongation of the zoites showed cytoplasm of uniform aspect, coloured red or blue with azocarmine and orange with Mallory's triple stain. In some cases on both sides of the elongation and at the rear of the spherical corpuscle some darker bands could be seen (Pl. IV, 14, 15).

The nucleus of about 3 μm in diameter, occupied the posterior end of the zoite and was surrounded by a clear area. It showed a small nucleolus (0.5 μm) and small peripheric granules of chromatin.

The average size of these germs was 9–11 μm × 4–5 μm, although some were observed measuring from 7 to 14 μm in length and 7 μm in width.

The smallest, generally stubby, showed a smaller terminal part.

(2) This type of zoite showed in the majority of the cases pointed ends and some were arrow-shaped at the front, as were the germs of Type I (Fig. 3, Pl. IV 13).

The cytoplasm contained very small granules that took on different colors (red, orange, blue) with Heidenhain's azocarmine. The nucleus, central or slightly towards the rear of the merozoite, had the size of 2 to 2.5 μm. Exceptionally, some of them were observed to have two nuclei. The nucleolus was small, but always visible.

The average size of these germs varied from between 6 to 10 μm in length and 3 to 5 μm in width.

(3) The smallest size, between 3 and 6 μm in length and 2.5 to 3 μm in width. They usually were pear-shaped with a round posterior end and a slightly poined front (Fig. 3, Pl. IV 13).

The cytoplasm showed polychromatic reactions with Heidenhain's azocarmine, sometimes had a uniform aspect, and at other times a granulated one. The nucleus, about 2 μm, was generally located in the middle of the zoite.

In the PAS staining, only the zoites of Type I showed positive granules (Pl. IV 16). The processes of PAS deamination did not affect the latter.

Discussion

The studies on parasitic protozoa of Enteropneusts are scarce and are based on isolated light microscope observations of different phases of the life cycle.

Material characteristics condition to a great extent this lack of information, due to the fact that certain experimental techniques, such as inoculation or incubation, used with other groups of animals, are not applicable here.

On Coccidia specifically, the studies are limited to the investigations of Leger and Duboscq (1917) and Benito (1977) in *Glossobalanus minutus*. The former described two species which they included in the genus *Eimeria*, one for hepatic *caeca*, *E.? beauchampi* and the other for epidermis, *E.? epidermica*. The generic attribution of both is still uncertain as the sporogony is unknown, which possibly is due to the cleansing operation to which the specimens are subjected for removing the sand from their digestive tract and carrying out the necessary histological techniques.

Then again, Leger and Duboscq regret not having ever observed, in spite of a high level of infestation, the merozoites in intestinal lumen. For this reason, they considered favorably the suggestions of Schellack and Reichnow (1913) on a purely epithelial propagation.

The present study demonstrate, however, that the infestation is extra-epithelial, with numerous infections germs being observed in the lumen of hepatic sacculations in three of the studied specimens, which, in view of their morphological and cytological characteristics, we have considered to be merozoites.

The majority of them were located on the ciliated layer, although others were found penetrating the epithelium or in its interior.

The diversity and agglomeration of merozoites in the lumen and the relatively low number of small schizozoites present in the adult schizont, led us to the assumption that the cycle of *Eimeria beauchampi* could have more than one generation of schizonts.

In the life cycles of the other types of *Eimeria*, the existence of various agamic generations has been demonstrated, differentiated by their morphology, location, number and size of merozoites, as recorded in the works of Marinček (1973), Senaud and Černá (1969), Tood et al. (1968 a), Tood and Hammond (1968 b, c), Musajev and Ismailov (1971), Norton (1967), Ankro et al. (1975), among others.

This hypothesis was favored by observing, in the epithelium of the front hepatic sacculations, schizogonic forms different from those described by Leger and Duboscq for *E. beauchampi* and designated in this study as Schizogony A. The larger size of the latter led us to consider that this was the cause of the merozoites that we described as

types I and II, assuming then that the merozoites of Type III arose from schozogony A. This thesis was supported by two factors: the existence of a larger number of merozoites of Types I and II on the epithelium occupied by groups of gamonts, and the scarcity of merozoites present in lumen of sacculations parasitized by small schizonts belonging to Type III.

Then again, it is possible that the difference observed between merozoites of Types I and II was due to the existence of sexual dimorphism, assuming that those of the first type were the cause of the macrogamonts, while those of second were the cause of the microgamonts. This was suggested by some authors, such as Perez (1903), and demonstrated by others, such as Siedlecki (1899), Bonnet-Eymard (1900) and Schaudin (1909). Nevertheless, it is possible that merozoites of Type II created a new generation of schizonts.

Some authors assume the bi- or multinucleate merozoites to be the source of gametogenesis (Senaud and Černá 1969), although there are other interpretations, such as those set forth in the works of Speer and Hammond (1971), Senaud and Černá (1969), Speer et al (1970), Danforth and Hammond (1972), Shah (1971), Ankron et al. (1975).

The binucleate merozoites observed in *Glossobalanus minutus* are very scare, and we consider them, in principle, as exceptional or abnormal phenomena.

Notwithstanding these deductions, there are reasons for considering schizogony B as that described by Leger and Duboscq for *Eimeria epidermica*. The shape of the schizonts, their location in the epithelium, the size and the distribution of the schizozoites, as well as their number in the terminal schizont, permit to establish a relationship between them. Those authors point out the epidermic character of this species in addition to the nuclei and the chromatoidsubstance. For all that, they state that they have observed multinucleate forms and "barrel" distributions in the rear part of the ciliated groove, as a result of which they suggest that *Eimeria epidermica* may be an intestinal parasite that propagates rapidly to the external tegument. Consequently, the presence of this species in the hepatic region may be considered possible.

Various hypothesis are suggested by this study which we hope to be able to clarify with new investigations:

(1) That *E. beauchampi* has more than one agamic generation.

(2) That the schizogonic stages observed in the front hepatic saccu-

lations belong either to the second generation of *E. beauchampi* or to the schizogony of *E. epidermica*.

(3) That both species constitute a sole *Coccidium*, because only the schizogony of *E. epidermica* is known.

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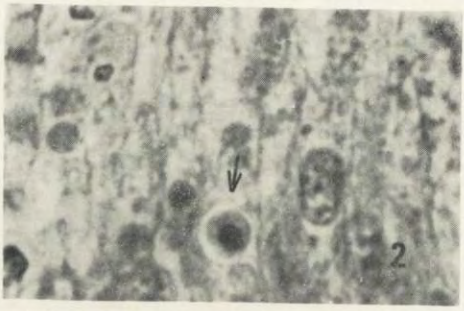
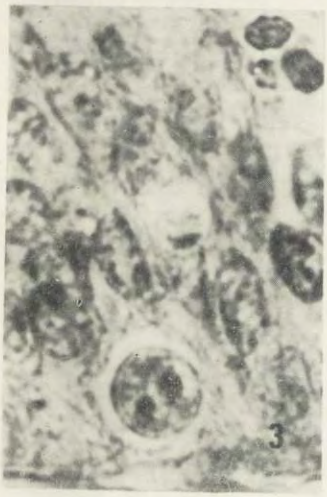
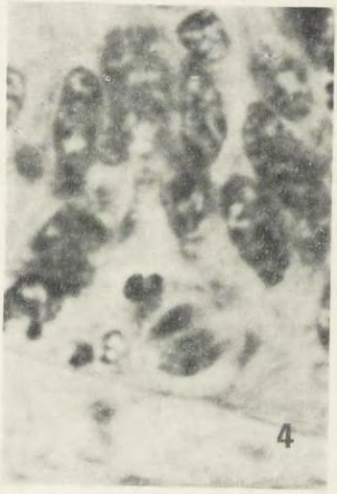
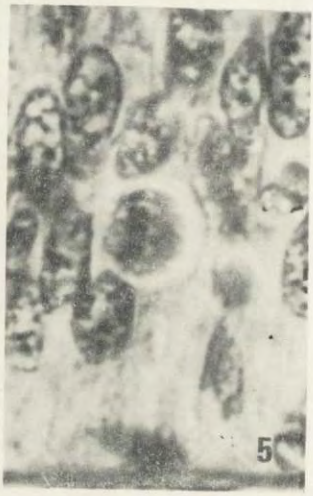
EXPLANATIONS OF PLATES I–IV

1: Merozoite lying free in the lumen of posterior hepatics sacculations. Heideenhain's azocarmine. × 1800
2-5: Stages of asexual development of *Eimeria beauchampi*
2: Trophozoite. Heidenhain's azocarmine. × 1800
3: Early schizont with two nuclei. Ninhidryne-Schiff. × 1800
4: Schizont containing mature merozoites. Ninhidryne-Schiff. × 1800
5: Mature schizont with many merozoites. Ninhidryne-Schiff. ×1800
6–10: Schizogony in the epithelium of anterior hepatics sacculations
6: Young schizont. Heidenhain's azocarmine. × 1800
7: Larger uninucleate schizont. Masson's triple stain. × 1800
8: Plurinucleate schizont. Ninhidryne-Schiff. × 1800
9: Plurinucleate schizont. Heidenhain's azocarmine. × 1800
10: Mature schizonts with many merozoites. Masson's triple stain. × 1800
11: Free merozoites on the epithelium surface. Heidenhain's azocarmine. × 720
12: Gamonts in the epithelium and free merozoites in the lumen of hepathiics sacculations. Heidenhain's azocarmine. × 720
13: Some types of merozoites on surface epithelium. Heidenhain's azocarminne. × 1800
14–15: Merozoite I (detalis). Mallory's triple stain. × 1800
16: Merozoites I with PAS positive granules. AB–PAS. × 1800









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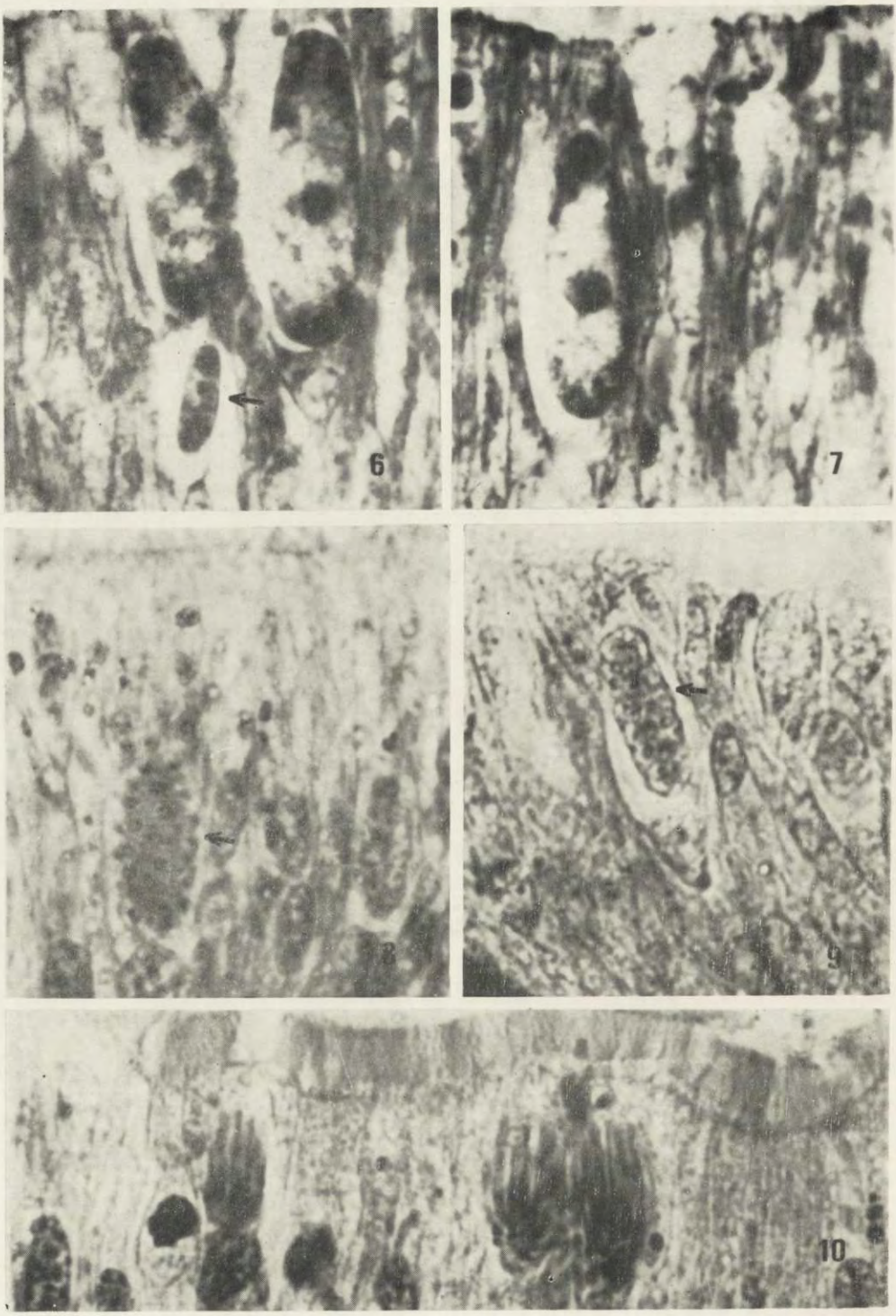

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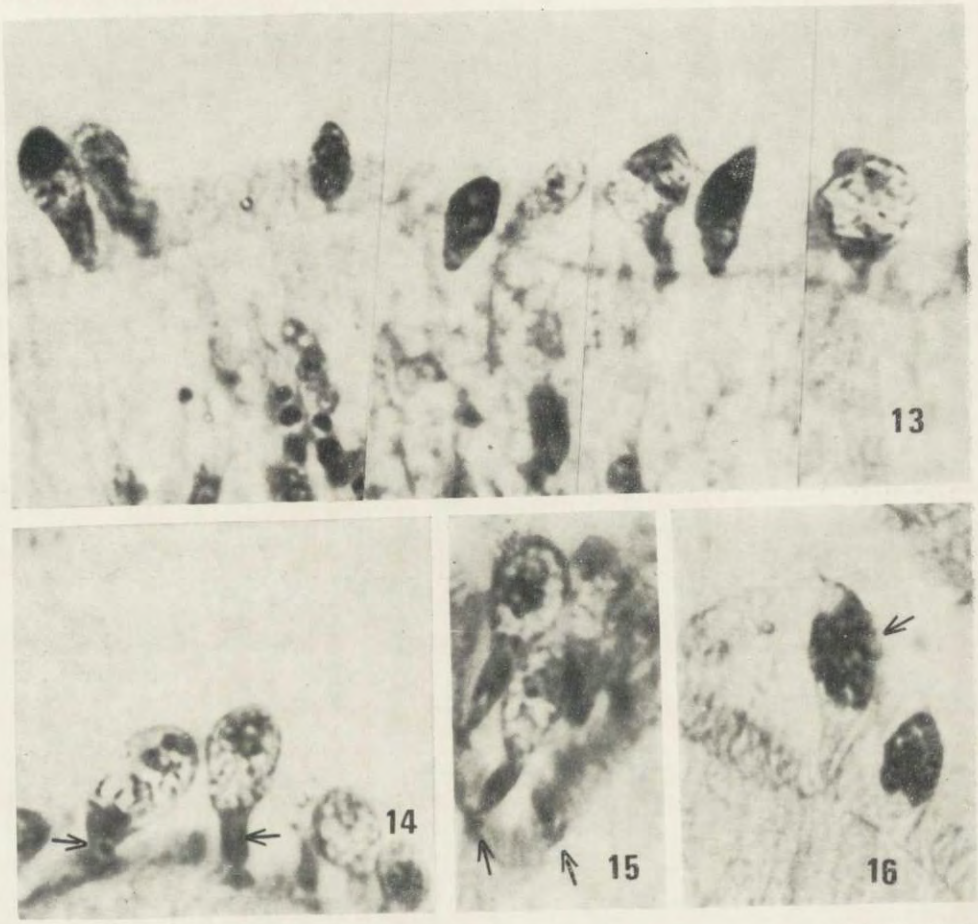

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Nouvelles observations sur les deux ciliés Oligotriches: *Halteria grandinella* (la morphologie et la morphogenèse) et *Strombilidium gyrans* (la morphologie)

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Synopsis. Avec l'emploi de la technique de Fernandez-Galiano (1976) nous décrivons des nouvelles données sur la morphologie, surtout de l'infraciliature et des systèmes fibrillaires de *Halteria grandinella* et de *Strombilidium gyrans*, et sur la morphogenèse de *Halteria grandinella*.

Dans les échantillons que nous prenons pour réaliser des études de l'écologie des protozoaires ciliés, nous avons rencontré deux espèces d'Oligotriches qui appartenaient aux genres *Halteria* et *Strombilidium*. Bien que ne tant comme dans les autres groupes de ciliés, les oligotriches ont été l'objet de plusieurs études (Corliss 1979), parmi lesquels on peut détacher celles de Fauré-Fremiet (1953, 1969), Fauré-Fremiet et Garnier (1970) et Grain (1972). Il existe, cependant, quelques aspects sur la morphologie et la morphologenèse de *Halteria grandinella* et sur la morphologie de *Strombilidium gyrans* inconnus que nous montrons ici.

Matériel et techniques

Les échantillons où existaient les deux espèces proviennent de la rivière Alberche, près de Madrid (Espagne). Les échantillons ont été enrichis par des grains de blé (2 grains/50 ml d'eau) et directement traités avec la technique du carbonate d'argent ammoniacal pyridiné (Fernandez-Galiano 1976). On a obtenu des photomicrographies des exemplaires impregnés.

Observations

La morphologie de *Halteria grandinella*

Halteria grandinella O. F. Müller, 1786 est à la famille *Halteriidae* Claparède et Lachmann, 1858, sous-ordre *Oligotrichina* Butschli, 1887, ordre *Oligotrichida* Butschli, 1887 (Kahl 1935, Corliss 1979).

Des exemplaires, d'aspect plus ou moins sphérique, mesurent approximativement 30 μm de diamètre. Le macronoyau allongé a 18 μm× ×10.4 μm (en moyenne). Le micronoyau, relativement grand et sphérique, mesure 3.2 μm de diamètre (Pl. I 1).

La ciliature somatique (CS) se compose de rangées de cinétosomes courtes, situées parmi le tiers entérieur du cilié et son équateur, légèrement inclinées par rapport à l'axe antéro-postérieur du cilié. Chaque cinétie a une longueur de 3.6 μm (en moyenne) et possède 6 paires de cinétosomes. Chaque cinétie somatique a une fibre commune formée par des microtubules en rideaux décrites par Grain (1972). Nous avons appelé cette formation, fibre microtubulaire somatique (FMS) (Pl. II 5).

La zone adorale de membranelles (ZAM) est formée par 16 membranelles; chacune de ces membranelles a une longueur en moyenne de 4.4 μm et un largeur en moyenne, à la base, de 0.76 μm. La membranelle possède 4 cinéties: la plus courte a 2–3 cinétosomes et 0.7 μm de longueur (en moyenne); près de cette cinétie il y a une autre de 2.8 μm de longueur en moyenne et elle a 7–8 cinétosomes; à côté et dans chaque membranelle il y a des autres deux cinéties de la même longueur (3.9 μm en moyenne), chacune avec 12–14 cinétosomes (Pl. I 1, 2).

Les membranelles adorales continuent par leur extrême gauche dans 6–8 membranelles qui forment la zone orale de membranelles (ZOM); elles sont plus assemblées que les membranelles adorales. Chacune de ces membranelles a une longueur de 2.3 μm (en moyenne) et une largeur, à la base, de 0.48 μm (en moyenne) (Pl. I 1, 2).

Dans la région orale existe une cinétie parorale (CP) de 7.8 μm de longueur en moyenne; il s'agit d'une cinétie simple formée par 18–22 cinétosomes (Pl. I 1, II 5).

La morphogenèse de *Halteria grandinella*

Par dessous de l'extrême postérieur des cinéties orales et à la distance de 2.72 μm (en moyenne) de cet extrême apparaît le primordium oral (PO). Cette ébauche est formée, au début, par un champ de cinétosomes qui se misent en ordre, postérieurement, en s'organisant par des ran-

gées simples et après doubles de cinétosomes. Cette organisation se réalise selon un gradient, dès la région la plus antérieure de l'ébauche à la plus postérieure, et de la droite à la gauche (Pl. I 3). Quand on montre la formation de rangées doubles de cinétosomes sur toute la longueur de l'ébauche il y a 21 premembranelles (en moyenne). Dans ce stade l'ébauche orale (PO) se situe en formant un angle aproximé de 20° par rapport à l'axe antéro-postérieur du cilié (Pl. I 4). Les membranelles prolifèrent et elles se séparent en donnant aux deux champs, adoral et oral, des membranelles. L'orientation reste quant au champ oral de telle manière que les membranelles s'étendent autour d'un axe qui suit en constituant un angle de 20° (en moyenne) par rapport à l'axe antéropostérieur du protère. On a observé que la cinétie parorale du opisthe apparaît près de la membranelle adorale la plus antérieure du primordium, par-ce qu'il est possible qu'elle soit provenante de la division d'une partie ou du total des cinétosomes de cette membranelle. Les cinéties somatiques se multiplient, pour se situer, après, dans leur lieu définitif à l'opisthe (Pl. II 5, 6).

La morphologie de *Strombilidium gyrans*

Strombilidium gyrans Schewiakoff, 1893 est à la famille *Strombilidiidae* Doflein et Reichenow, 1929, sous-ordre *Oligotrichina* Butschli 1887, ordre *Oligotrichida* Butschli, 1887 (Kahl 1935, Corliss 1979).

Les exemplaires, d'aspect plus ou moins sphérique après la fixation utilisée, ont 75 μm de diamètre (en moyenne), mais *in vivo* ils ont une forme allongée avec l'extrême postérieur du corps aiguisé, en mesurant alors 85–90 μm de longueur. Le macronoyau, allongé et granulaire, grossi à ses extrêmes, est situé dans le tiers antérieur du cilié et il a une grandeur de 54 μm de longueur et de 12.5 μm de largeur à sa part plus grossie (en moyenne). Le micronoyau, bien évident, plus ou moins sphérique, a 5.8 μm de diamètre (en moyenne) (Pl. III 7).

La ciliature relationnée avec la région orale est située à la partie la plus apicale du cilié où on voit une zone adorale de membranelles (ZAM), une zone orale de membranelles (ZOM) et la cinétie parorale (CP).

La zone adorale de membranelles (ZAM) constitue un circle ouvert uniquement par la région qui correspond au cytostome, aux membranelles orales et à la cinétie parorale. Cette ceinture de membranelles (ZAM) a une longueur de 70 μm (en moyenne), à la base des membranelles, et est formée par 29–30 organelles. Chaque membranelle adorale a 14 μm de longueur (en moyenne) et est formée par 3 cinéties, une

plus courte composée par 46 cinétosomes (en moyenne) et deux plus longues avec 52 cinétosomes (en moyenne) chacune (Pl. III 8, 9, 10). Nous avons observé qu'il existe une fibre commune à la zone adorale de membranelles et située selon l'axe principal de cette structure; nous avons appelé cette fibre, fibre sousmembranellaire (FSM) (Pl. III 9).

La zone orale de membranelles (ZOM) est formée par 4 membranelles dont la longueur est variable; la membranelle la plus allongée est près de la ZAM et a 4.48 μm de longueur (en moyenne); la cinétie la plus courte et postérieure a 2.2 μm de longueur (en moyenne). Chacune de ces membranelles possède 3 cinéties, approximativement avec le même nombre de cinétosomes qui varie dès 12 à la membranelle la plus antérieure, jusqu'à 5 à la plus postérieure (en moyenne) (Pl. III 7).

La cinétie parorale (CP), simple et très courte par rapport à la ZAM, a 9 μm de longueur (en moyenne) et elle se compose de 40 cinétosomes (en moyenne) en formant une seule rangée.

Discussion

La morphologie de *Halteria grandinella*

Quant à la ciliature somatique, le nombre de cinétosomes constitués dans les paires que nous avons observées ne coïncide pas avec les nombres décrits par Grain (1972) (6–12 cils par chaque cinétie), par Tamar (1965, 1968) (4 soies pour chaque cinétie) et par Fauré-Fremiet (1953) (4 soies simples ou doubles pour chaque cinétie). Nous avons compté 16 membranelles adorales par rapport aux 15 de Grain (1972) et en accord avec le nombre de Fauré-Fremiet (1953) et de Tamar (1965, 1968). Chaque membranelle adorale est formée par 4 cinéties et non par 3 (Grain 1972) et les membranelles orales sont 6–8 (8 d'après Tamar 1965) et non 10 comme a décrit Grain (1972), cependant, en accord avec ce dernier auteur les compositions cinétiques des membranelles orales correspondent à notres observations. Chaque des membranelles adorales a une longueur moyenne de 4.4 μm en comparaison avec 5.5–7 μm de Tamar (1974).

La morphogenèse de *Halteria grandinella*

Szabô (1953) a décrit l'origine des membranelles adorales et orales à partir d'une rangée simple de primordiums. A notre avis, l'origine de ces membranelles est un champ cinétosomique qui apparaît près de l'extrême postérieur de la zone orale de membranelles.

Il faut détacher que, en partie, cette morphogenèse est semblable à celle décrite par Fauré-Fremiet (1953), sauf que nous n'avons pas observé aucun migration du primordium oral dès le stade dans lequel on voit les premembranelles bien différenciées, à partir de la formation de cinéties doubles. La situation reste la même avec l'axe incliné 20° (en moyenne), avec l'axe antéro-postérieur du protère, jusqu'aux derniers stades de la morphogenèse (Fig. 9, Fauré-Fremiet 1953) et seulement les cinéties somatiques migrent pour obtenir sa position définitive avant la scission.

La morphologie de *Strombilidium gyrans*

Nous considérons le travail le plus actuel sur cette espèce (Deroux 1974) et la disposition de la ciliature somatique est la même que celle décrite par cet auteur. Cependant, dans ce travail il n'y a pas de suffisantes données numériques qui peuvent se comparer avec les nôtres. D'après Deroux la frange adorale se compose de 22–23 membranelles dont deux sont typiquement orales et plus courtes que les autres. Nous avons observé que le nombre de membranelles adorales est 29–30, mais les quatre membranelles les plus postérieures ont des segments courts qui présentent une orientation différente au reste de la membranelle et qui constituent la zone orale de membranelles, adjacente au cytostome.

Conclusions

La morphologie et la morphogenèse de *Halteria grandinella*

Nous décrivons, entre des autres nouvelles, les caractéristiques numériques du macronoyau, des membranelles orales et adorales, de la cinétie parorale et des cinéties somatiques, quelque différentes à celles montrées jusqu' à maintenant. Quant à la morphogenèse, nous montrons, peur la première fois, les données photographiques des stades de ce procès, seulement décrit jusqu' à maintenant par les dessins (Fauré-Fremiet 1953). D'autre côté, nous avons observé qu'il n'existe pas translocation secondaire des membranelles adorales et orales dans la morphogenèse et que par ce fait on ne traite pas d'une bipartition énantiotrope mais, en tout cas, d'une bipartition hétéropolaire, avec un angle bien defini entre les axes antéro-postérieurs du protère et du opisthe qui reste ainsi jusqu' à la scission.

La morphologie de *Strombilidium gyrans*

L'emploi de la technique du carbonate d'argent permet décrire, peur la première fois, des aspects de la composition cinétosomique et des mesures de la ciliature dépendante de la région orale; ces données sont relatives à des membranelles adorales et orales et à la cinétie parorale. On montre aussi des données quantitatives du macronoyau, du micronoyau et du cilié en général. On a observé l'existence d'une fibre sous-membranellaire commune aux membranelles adorales.

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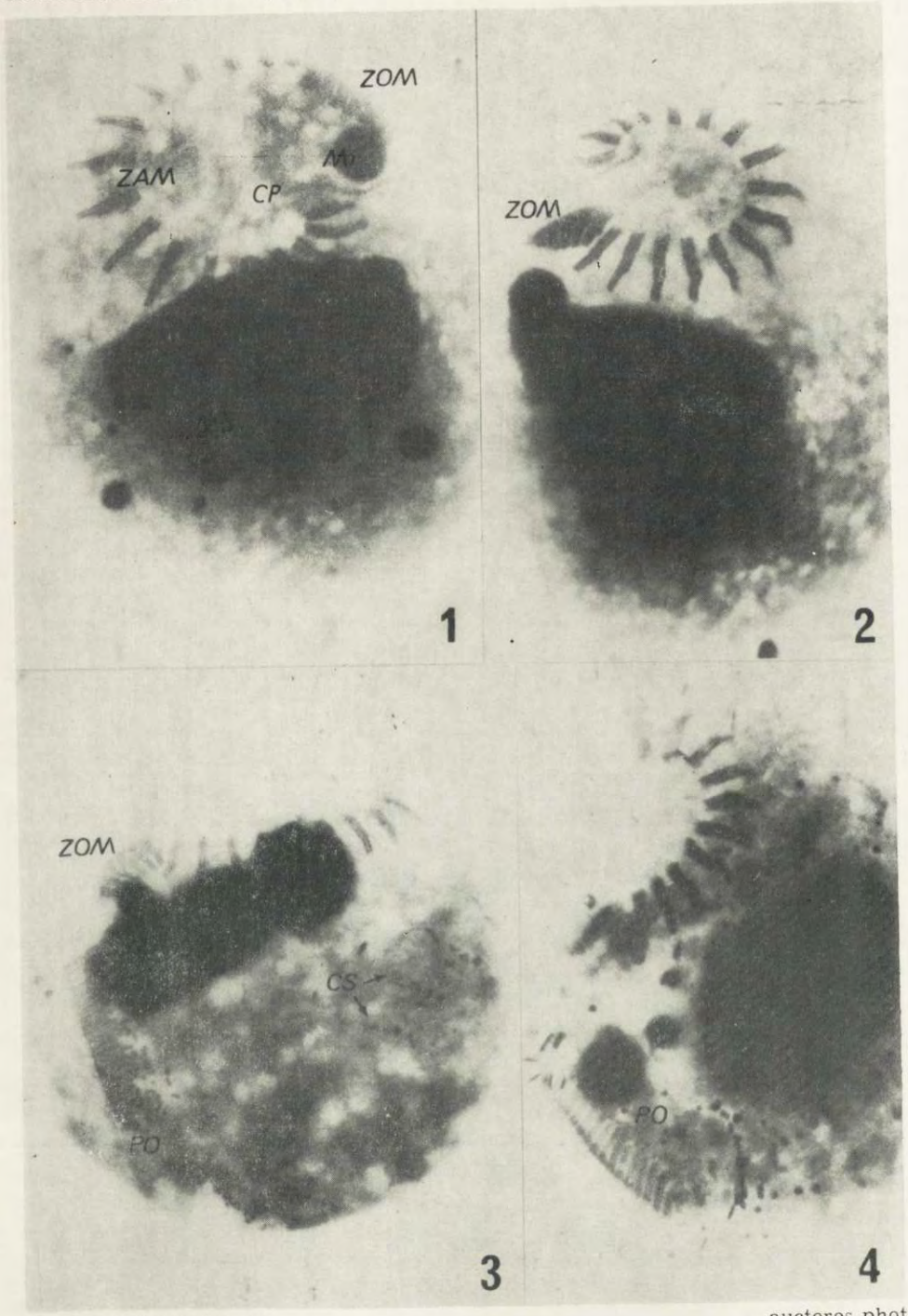
EXPLICATION DES PLANCHES I–III

1: L'aspect général d'un exemplaire de *Halteria grandinella* avec la zone adorale de membranelles (ZAM), la zone orale de membranelles (ZOM), la cinétie parorale (CP), le macronoyau (Ma) et la micronoyau (Mi)
2: Un autre aspect de *H. grandinella* montrant la ZOM
3: Stade de la morphogenèse de *H. grandinella*. On montre les cinéties somatiques (CS) et le primordium oral (PO)
4: *H. grandinella*. Stade avec les doubles rangées de cinétosomes dans le PO
5: *H. grandinella*. Vers la fin de la morphogenèse on observe les structures ciliaires du opisthe déjà formées, Les cinéties somatiques du protère (CSP) et du opisthe (CSO) et la fibre microtubulaire somatique (FMS)
6: *H. grandinella*. Un autre aspect de la fin de la morphogenèse. On observe les deux micronoyaux de chaque tomite
7: Vue générale de *Strombilidium gyrans*. La zone orale de membranelles (ZAM) et le macronoyau (Ma)
8: *S. gyrans*. La ciliature orale et le micronoyau
9: *S. gyrans*. La cinétie parorale (CP) et la fibre sousmembranellaire (FSM). On observe aussi la composition de la ZAM
10: *S. gyrans*. Détail des membranelles adorales (m)



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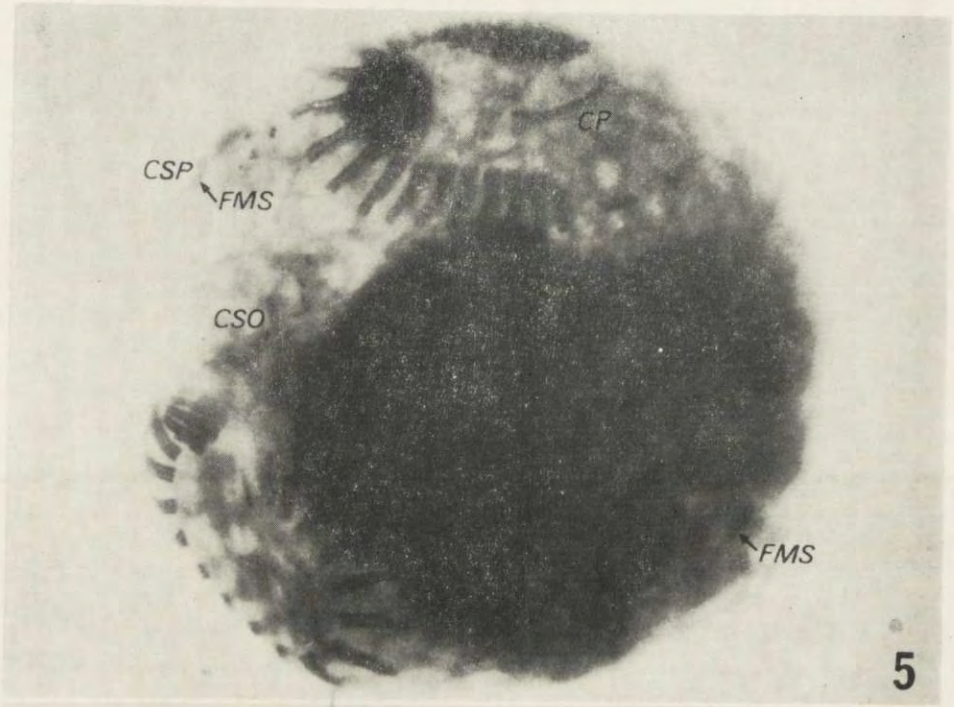

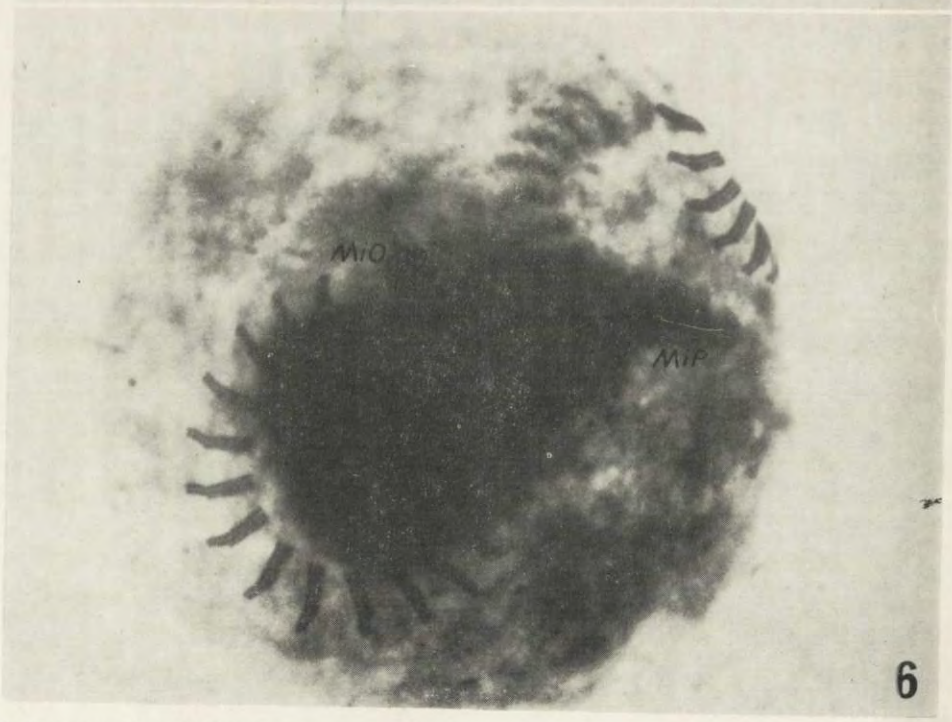

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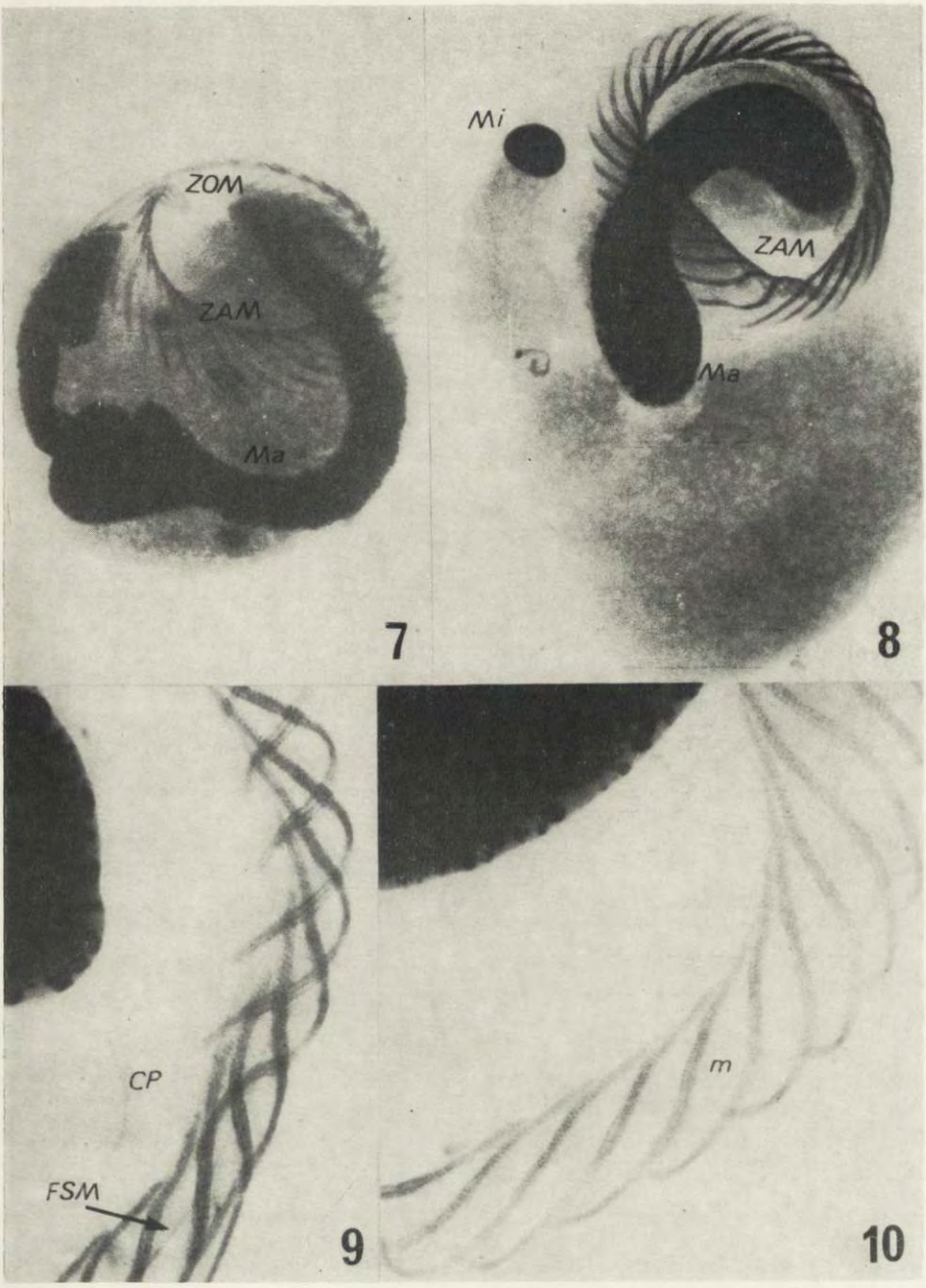

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Myxosporidian *Sphaeromyxa dighae* sp. n. (*Myxozoa: Myxidiidae*) from the gallbladder of *Hilsa ilisha* (*Clupeidae*)

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Synopsis. The paper describes the myxosporidian *Sphaeromyxa dighae* sp. n. from the gallbladder of *Hilsa ilisha*, caught off the Digha coast of the Bay of Bengal, West Bengal, India. The present species has been compared with its other related species.

Previous reports (Chakravarty 1939, 1943) reveal that the two myxosporidan, *Ceratomyxa hilsae* Chakravarty, 1939 and *Zschokkella ilüshae* Chakravarty, 1943 have been described from the gallbladder of fresh water fish *Hilsa ilisha*. However, a new myxosporidan of the genus *Sphaeromyxa* Thelohan, 1895 has been detected in the gallbladder off the fish *Hilsa ilisha* from saline water (Digha coast of the Bay of Bengal) during the present investigation.

Material and Methods

All autopsies were made on fridged fish (kept in ice bucket) collected from the fishermen on the Digha coast of the Bay of Bengal. The observations were made from wet smears and also from dry smears stained with Giemsa after fixation in absolute methanol. Diagrams were made with the help of a camera lucida (mirror type). All the measurements are given in micrometer (μm) with mean, and the range within parenthesis.

Observations

Sphaeromyxa dighae sp. n.

Trophic stages — not seen. However, from the disposition of the two spores side by side facing each other it seemed that the trophozoite was disporous.

Spore — coelozoic, large and broad, bent in the middle forming an angle of 140 degrees in valvular view (Fig. 1 *1*). Shell valves smooth, elongated and curved. Each arm of the spore had a very sharp terminal bent which was beyond the polar capsule in oblique view (Fig. 1 *2*).

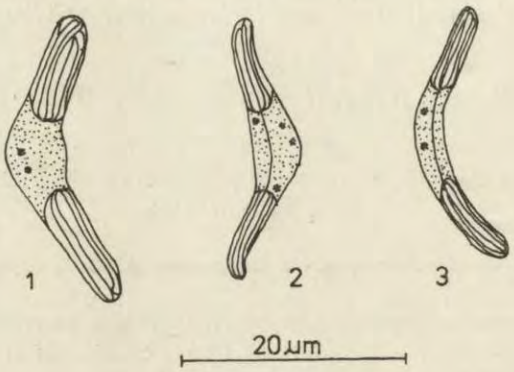

Fig. 1, *1–3*. Spores of *Sphaeromyxa dighae* sp. n., *1* — A spore in valvular view, *2* — A spore in oblique view, *3* — A spore in sutural view

However, the spore appeared arched in sutural view (Fig. 1 *3*). The suture was curved. The polar capsules were two — one on each end of the spore, subterminal and ellipsoidal. The capsular nucleus was usually found at the base of each capsule. The polar filament was ribbon-like, 4–5 coils in each capsule and parallel to the long axis of the spore.

The coarse and binucleate sporoplasm completely filled the extracapsular cavity of the spores between the two capsules. The capsules in each spore were almost equal. The valvular nucleus was indistinct.

Dimensions:
Spore (based on 31 spores stained with Giemsa):
Length — 23.82 (21.0–25.0)
Breadth — 3.33 (2.8–4.5)
Polar capsule (based on 63 capsules stained with Giemsa)
Length — 8.43 (7.0–10.0)
Breadth — 2.32 (1.8–3.0)
Infection locus: Gallbladder (bile)
Incidence: 1 parasitized out of 5 fishes examined
Host: *Hilsa ilisha*
Locality: Digha coast of Bay of Bengal, West Bengal, India

Remarks: In having smooth and curved spores, the present myxosporidan apparently resembles *Sphaeromyxa reinhardti* Jameson, 1929 from the gallbladder of *Engraulus mordax* Girard, *S. gibbonsia* Noble, 1939, from the gallbladder of *Gibbonsia elegans elegans* and *G. metzi*

Table 1

Name of the parasite (Name of the host)	Spore	
	Length	Breadth
Sphaeromyxa reinhardti (*Engraulus mordax*) Jameson, 1929	21.25–23.3	3.75–5.0
S. gibbonsia (*Gibbonsia elegans elegans*) Noble, 1939	27.0	5.2
S. theraponi (*Therapon jarbua*) Tripathi, 1951	19.8	5.4
S. sabrazesi (*Motella* sp. *Hippocampus* sp.) Laveran et Mesnil (cited from Kudo, 1966)	22.0–28.0	3.0–4.0
Sphaeromyxa dighae sp. n. (*Hilsa ilisha*)	(21.0–25.0) 23.82	(2.8–4.5) 3.33

This table shows the mensural variations of spores of the related species of *Sphaeromyxa dighae* sp. n.

Hubbs, *S. theraponi* Tripathi, 1951 from the gallbladder of *Therapon jarbua* and *S. sabrazesi* (K u d o 1966) from the gallbladder of *Hippocampus* sp. *Motella*, sp. and others. However, none of these *Sphaeromyxa* spp. possess the characteristics of the present species such as 140 degree angle between the two arms, arms with round ends, subterminal polar capsules, sharp terminal bent beyond the polar capsule of each arm, mensural variations (Table 1) and its host *Hilsa ilisha*. We, therefore, believe it is a new species for which the name *Sphaeromyxa dighae* sp. n. is proposed after the locality of the host.

M a t e r i a l: Syntypes on slide No. MGH-9, deposited in the Department of Zoology, R. B. College; later will be deposited to the National Collection of the Zoological Survey of India, Calcutta.

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Histopathology of a Microsporidian Infection Caused by *Plistophora legeri* (Paillot) in *Lobesia botrana* Den. et Schiff. (*Lepidoptera, Tortricidae*)

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Synopsis. A microsporidian *Plistophora legeri* causes a general infection in larvae, pupae and adults of *Lobesia botrana*. Pathological changes caused by this parasite in various tissues were described. The protozoan infects male and female gonads and is transovarially transmitted. Since this microsporidian infects compound eyes and brain it may affect the behaviour of adult moths.

Paillot (1941) in the original description of *Plistophora legeri* (= *Mesnilia legeri*) briefly mentioned that this microsporidian caused a general infection in *Clysia ambiguella* Hbn. Lipa (1981a, b) while redescribing and reclassifying this protozoan reported that this parasite caused a general infection in *Lobesia botrana* Den. et Schiff. In this paper the results of detaild studies are presented on pathological changes in larvae, pupae and adults of *L. botrana* caused by *P. legeri*.

Material and Methods

Larvae, pupae and adults of *L. botrana* used for this study originated from the laboratory rearing maintained at the Station of Zoology INRA in Montfavet. Some larvae were collected in a field population of *L. botrana* in Courthezon (Vaucluse county), France.

Grosspathology was studied in insects dissected in water and various organs and tissues were examined under the dissecting microscope.

For histological studies insects were fixed in Carnoy's fluid, 24 h washed in

100% ethyl alcohol, transferred three times through butyl alcohol and embedded into paraplast. Sections were cut 4–5 μm thick, and stained according to Vago and Amargier (1963) method. Some insects were fixed in Bouin's fluid washed in ethyl alcohol, transferred through methylbenzoate and benzene to paraffin, cut to sections 6–8 μm thick and stained with Giemsa's solution 0.25% for 24 h.

Results

Grosspathology

There are no clear external symptoms of infection caused in adults, pupae or lightly parasitized larvae. However, in heavily infected larvae abnormally whitish appearance of the ventral side can be noticed. This is a result of a huge number of spores present in midgut epithelium and white intestine is visible through cuticule.

Infected larvae are not as active as the healthy ones. In many cases their bodies are shortened and their length does not correspond to size of head capsule. Therefore such larvae look like they would be one or two instar younger.

When infected larvae are dissected white-coloured organs, containing huge number of spores, are easily noticed with or without a dissecting microscope. When different tissues of infected insects are microscopically examined spores grouped in pansporoblasts are easily noticed in various tissues: silk glands (Pl. I 1–2), gonads (Pl. I 3), eggs (Pl. I 4) and others.

Histopathology

Midgut epithelium

The midgut epithelium of healthy larvae is thin and consist one layer of cells. The nuclei of healthy cells are medium size and have a moderate number of karyosomes.

In the weakly infected larvae only some cells are infected and the one layer of cells is preserved. However, the heavily infected midgut epithelium has a quite different appearance. Spores of *P. legeri* occur in great number in cell cytoplasm, which proliferate toward the gut lumen and some type of nodules are formed (Pl. II 5). In most cases the size of infected cells greatly increases due to hypertrophy and presence of great number of spores. The size of nuclei of infected cells increases as well as the number of karyosomes.

In the gut lumen of the infected larvae a great number of spores

may be noticed. This is evidently due to the rupture of infected cells and due to abnormally intensive regeneration processes in the infected midgut epithelium. Heavy vacuolization is frequently seen in strongly infected epithelium.

In many pupae the midgut epithelium was the most heavily infected organ and its cells were completely filled with spores.

Silk gland

The silk glands are frequently and heavily infected with *P. legeri*. Cells of glandular as well as ducted regions are filled with numerous spores located in the cell cytoplasm.

Malpighian tubules

Walls of normal Malpighian tubules are thin and cells have elongated nuclei. On the other hand tubules or their parts infected with *P. legeri* are strongly hyperthrophied or swollen due to the presence of great number of spores (Pl. II 6). Nuclei of heavily infected cells are not well seen and some of them degenerate. Walls of infected tubules are 2–3 times thicker than of normal tubules. Lumen of heavily infected tubules is so greatly reduced that obviously such tubules do not function properly.

Hypodermis

Hypodermal cells of larvae are frequently and heavily infected by *P. legeri*. A frequent proliferation of hypodermal cells is observed which form nodules toward the body cavity. Such nodules contain great number of spores of *P. legeri*.

Hypodermal cells in adult moths are also frequently infected.

Fat body

Normal fat body cells examined on sections are seen as blue-grey net with large nuclei. Spores of *P. legeri* are well seen in form of pansporoblasts and can be easily recognized from large elipsoidal proteinaceaus bodies present in fat body cells (Pl. II 7). Fat body heavily infected looses its normal structure and its cells became ruptured what is well seen on electron micrograph (Pl. III 9).

Muscles

Muscles of larvae, pupae and adults are frequently infected with *P. legeri*. In some cases only focal infection is observed and single pansporoblasts are present within muscle bundles (Pl. II 8). However, in many cases large fragments of muscles are heavily attacked.

Infection of longitudinal and round muscles of larval gut is very frequently observed.

Gonads

Male and female gonads in adult moths are frequently infected with *P. legeri*. In case of male gonads the pathogen is developing in this part of testis where spermatocytes are formed as well as in this part where spermatids mature. In the heavily infected gonads deeply stained granules are frequently seen.

Spores and pansporoblasts are frequently observed in spermatophores which indicate that this parasite can be transmitted with sperm to healthy females during copulation.

In infected female gonads the follicular epithelium shows striking changes (Pl. III 10). The cell nuclei become hypertrophied or degenerated. The eggs become infected with *P. legeri* when they are still in ovarian tubes (Pl. III 11). Some eggs contain a great number of spores of *P. legeri* which clearly indicates that this parasite is transmitted transovarially (Pl. IV 12).

Brain

In several adult moths brain was attacked by the parasite. Development of *P. legeri* and its spores were observed among external part of the brain formed by nerve cells (Pl. IV 13). Acellular, central part of brain was not attacked.

Compound eyes

Compound eyes (Pl. IV 14) of adults have a complicated structure. Spores of the parasite were observed in the rhabdom region and in retinal cells which are located at the basal part of the ommatidium among sensory cells of the ommatidia (Pl. IV 15).

Discussion

As indicated by Lipa (1981 a, b) *Plistophora legeri* attacks various tissues of all developmental stages of *L. botrana* and causes a high mortality in populations of its hosts. This was recently confirmed by Deseo et al. (1981).

A great majority of histopathological studies of microsporidian infections were made in insect larvae and only a few in adult insects.

P. legeri infects male and female gonads of adult moths of *L. botrana* and is congenitally and transovarially transmitted. Since this microsporidian infects compound eyes and brain it may affect the behaviour of adult moths. The changes in behaviour of adult moths due to microsporidian infections may be more important than it is generally recognized. As it has been shown by Lalanne-Cassou and Percy (1976) *P. legeri* infects pheromone glands of *L. botrana*. Such glands may not produce pheromones in a normal quantity and infected females may not be as attractive for males as healthy females.

The above data indicate that besides of host mortality also other effects of the parasitic microsporidians on their hosts should be taken into account especially when impact of parasites on hosts' populations is considered.

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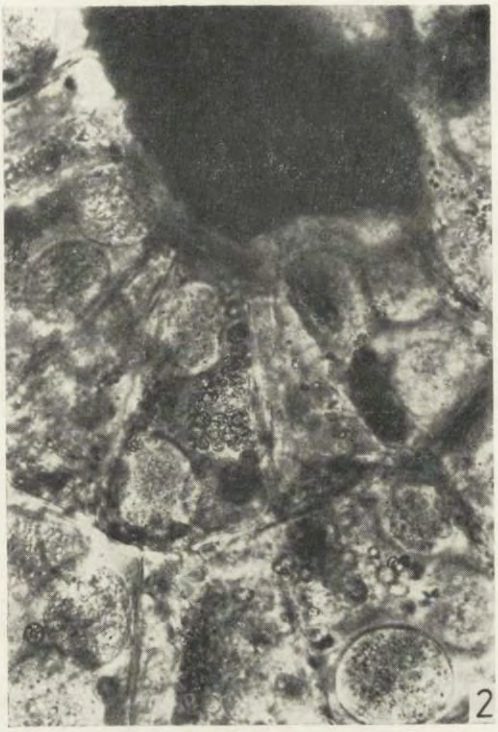
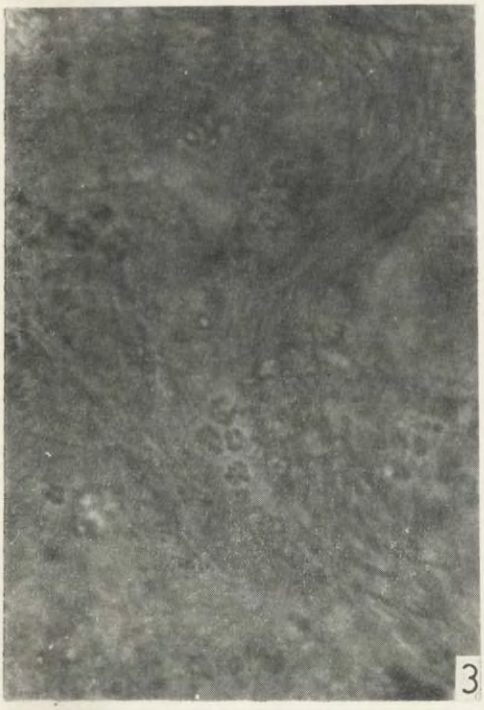
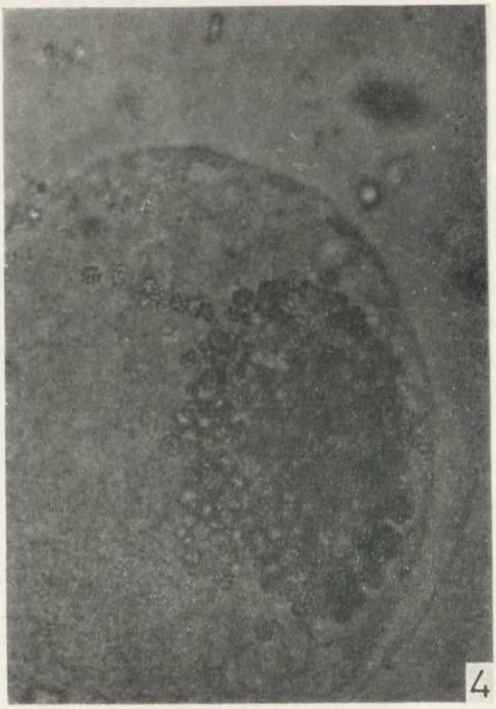
EXPLANATION OF PLATES I–IV

1: Spores in epithelial cells of ductal part of silk gland (280 ×)
2: Spores in pansporoblasts in squeezed secretory region of silk gland (110 ×)
3: Spores in pansporoblasts in squeezed male gonad and spermatophores, sperm flagella well seen (450 ×)
4: A great number of pansporoblasts within an egg. (280 ×)
5: Cross section through midgut epithelium of larvae with well seen spores in pansporoblasts (PS) and nuclei (N) of cells (280 ×)
6: Heavily infected Malpighian tubules, S-spores (1100 ×)
7: Pansporoblasts in fat body (1100 ×)
8: Groups of pansporoblasts (PS) in muscles (1100 ×)
9: Ultrathin section through destroyed fat body filled with spores: electron micrograph (7500 ×)
10: Cross section through gonad of the female moth, O — ovarioles, S — spores in pansporoblasts (280 ×)
11: Spores in pansporoblasts within the egg yolk, enlarged portion of phott. 10 marked S (1100 ×)
12: Cross section through egg with seen spores in pansporoblasts (1100 ×)
13: Cross section through brain of infected adult moth, S — spores, N — nuclei of neurons (280 ×)
14: Cross section through head of adult moths with seen compound eye (upper part) and brain (lower part) (280 ×)
15: Spores (S) seen in the rhabdom and retinal cells of ommatidia (1100 ×)







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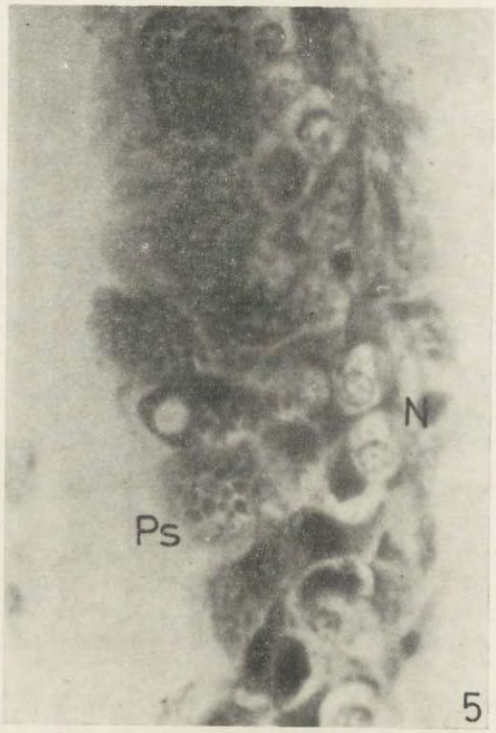

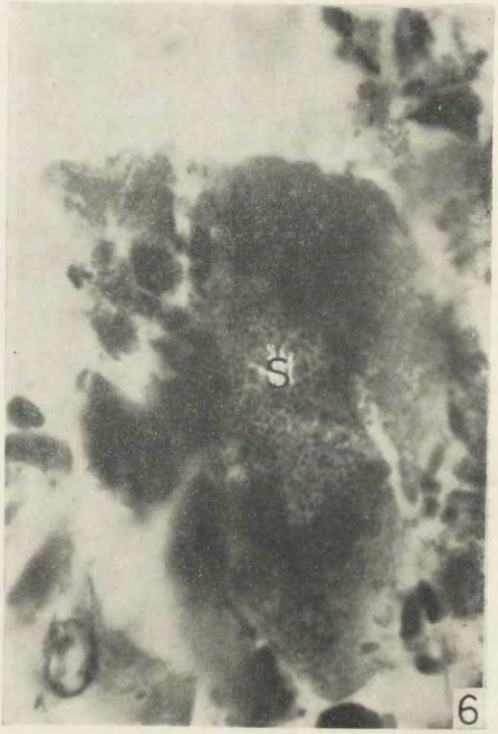

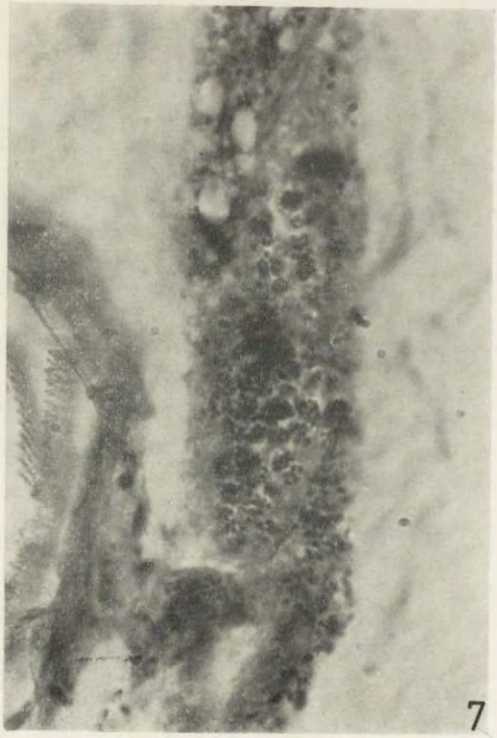

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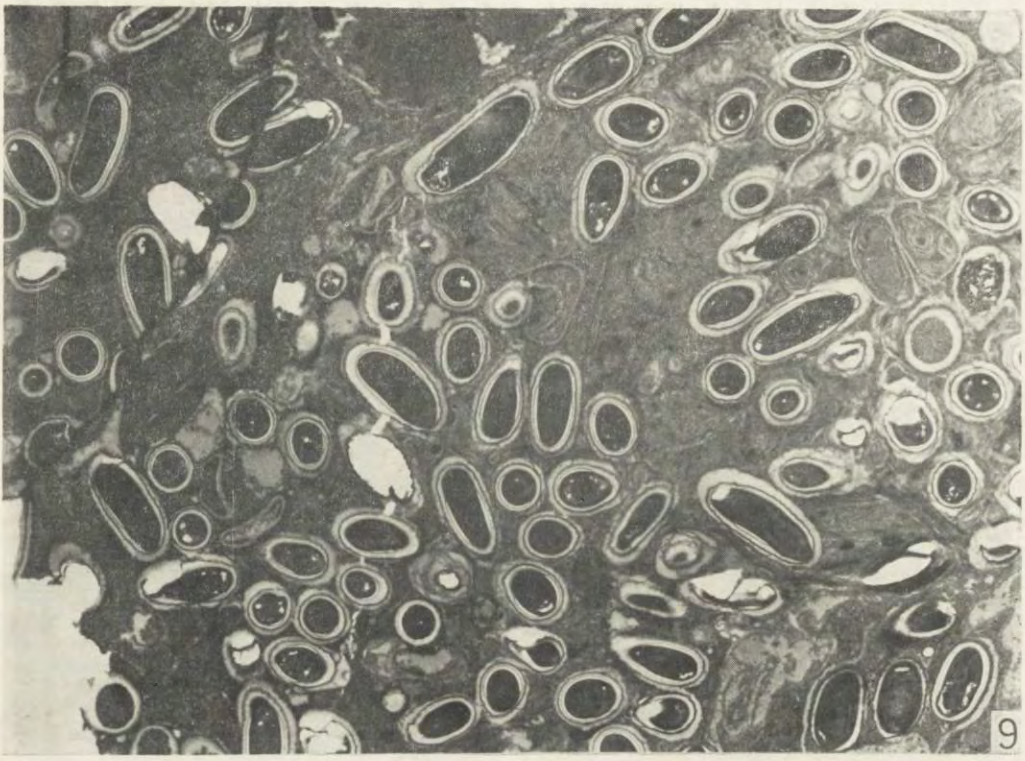

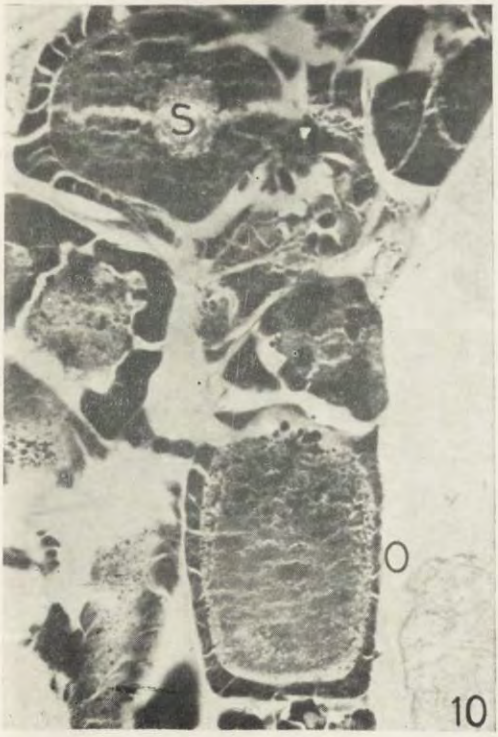

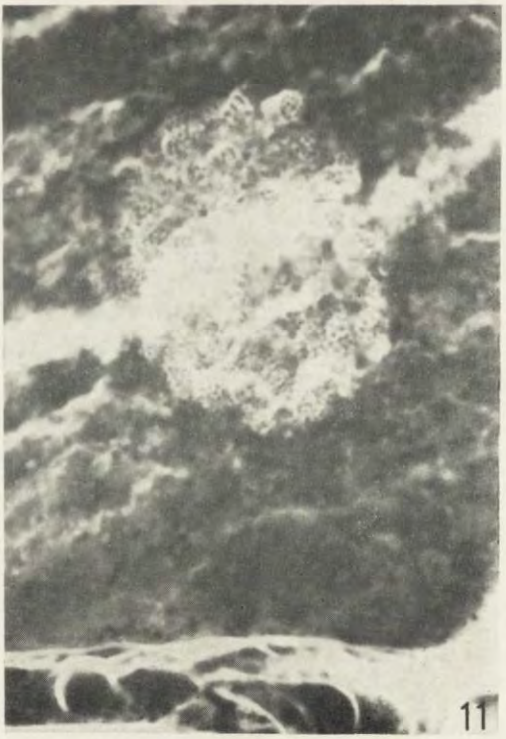

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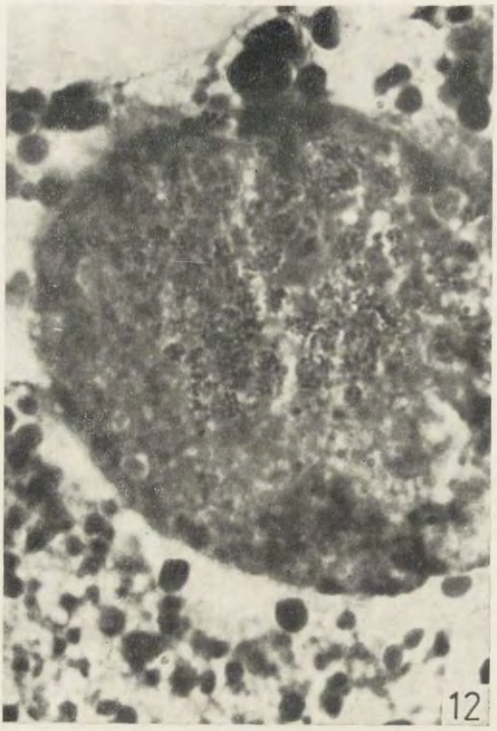

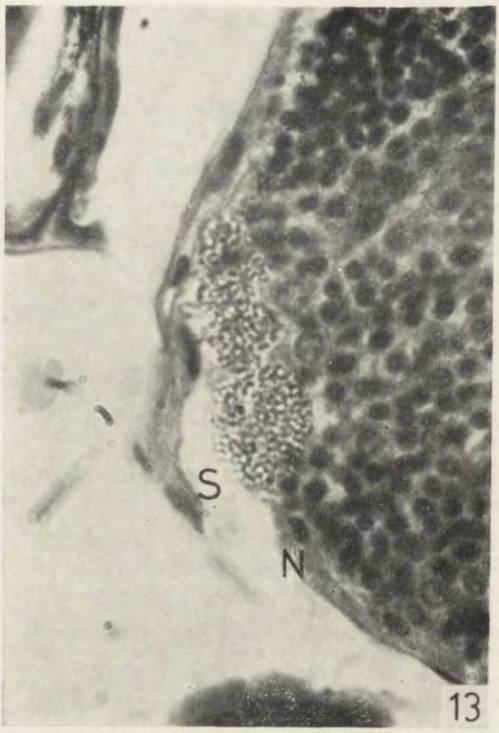

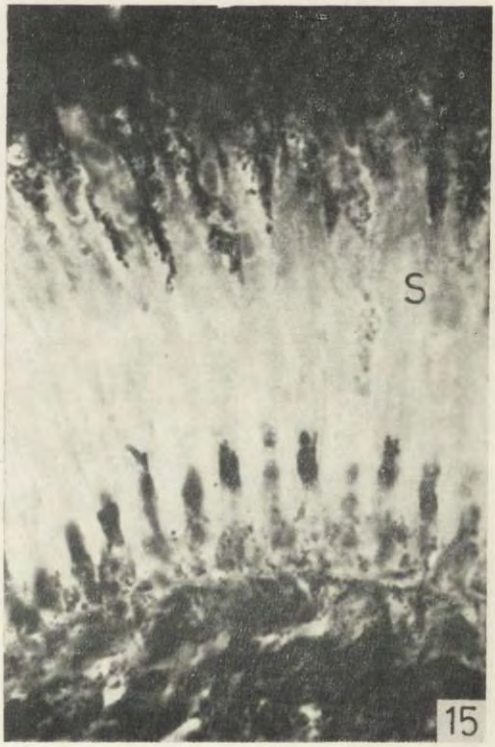

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A Comparison of Changes in the Volume of Microsporidian and Neogregarine Spores Using Various Methods of Preparing Spores for Inspection with the Scanning Electron Microscope

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Synopsis. Spores of both the neogregarine *Mattesia dispora* Naville, 1930 parasitic in larvae of *Plodia interpunctella* Hbn. and the microsporidian *Nosema plodiae* Kellen et Lindégren, 1969 recovered from larval *Mamestra brassicae* L., were prepared by eight different methods for the purpose of inspecting their surfaces and disclosing changes in their volume with the scanning electron microscope. Method No. 2 (without fixation; lyophilization) was found most satisfactory for examining the structure of the spore surface, although collapse of the spore wall changed considerably the volume of the spore (about to 1.64×10^{-10} $cm^3 = 21.9\%$ of the volume of a fresh spore of *M. dispora*, and $5.4 \times \times 10^{-12}$ $cm^3 = 49.5\%$ of the volume of a fresh spore of *N. plodiae*).

The result of an electron-microscopic study is greatly dependent on the method used for the preparing biological material. This applies particularly to surface structures to be examined with the scanning electron microscopes. So far microsporidian spores have only been treated with different preparation methods for inspection with the SEM (Žižka 1977, 1978a, Vávra and Barker 1977, 1980). SEM studies on the surface of trophozoites and gametocytes of eugregarines were made by several authors (Vávra and Small 1969, Heller and Weise 1973, Hildebrand and Vinckier 1975, and others), but the surface structure of spores has suffered from neglect (e.g., Åbro 1976). A possible explanation of this disinterest in the latter might have been deformations of the spore surface brought about by the use of the various methods.

Therefore, we designed eight methods in order to disclose the most

suitable one for the preparation of spore material for the SEM. In an earlier paper (Žižka 1978 b) we used three different methods for the preparation of spores of *M. dispora*, but did not evaluate the volume of spores or their morphology. Therefore, in the present paper, we compare changes on the volume in dependence on the morphology of the spores prepared with different methods for the SEM, using spores of the neogregarine *Mattesia dispora* Naville, 1930 and those of the microsporidian *Nosema plodiae* Kellen et Lindégren, 1960.

Material and Methods

Spores of *M. dispora* and *N. plodiae* obtained from *Plodia interpunctella* Hbn. and *Mamestra brassicae* L. were purified by a method suggested by Hostounský (1981) and prepared for the SEM with eight different methods:

(1) Air-drying of specimens rinsed in distilled water.

(2) Freezing of specimens in liquid nitrogen and drying in a high-vacuum desiccator at 2×10^{-5} Torr.

(3) Fixation of specimens with 1% OsO_4 for 3 h rinsing in distilled water and air-drying.

(4) Fixation with 1% OsO_4 for 3 h, rinsing in distilled water, freezing in liquid nitrogen and vacuum-drying (2×10^{-5} Torr).

(5) Fixation with 1% OsO_4 for 3 h, rinsing in distilled water, dehydrating with three changes of ethanol: 70% for 15 min, 96% for 15 min, 100% overnight, air-drying.

(6) Fixation with 6% glutaraldehyde for 3 h and with 1% OsO_4 for 3 h, rinsing in distilled water, air-drying.

(7) Fixation with 6% glutaraldehyde for 3 h and with 1% OsO_4 for 3 h, rinsing in distilled water, freezing in liquid nitrogen, vacuum-drying at 2×10^{-5} Torr.

(8) Fixation with 6% glutaraldehyde for 3 h and 1% OsO_4 for 3 h, rinsing in distilled water, dehydrating with 3 changes of ethanols (70% for 15 min, 96% for 15 min, 100% overnight), air-drying.

The method used for the freeze-drying (lyophilization) of our specimens was similar to that suggested by Nermut et al. (1972) for virus material and performed on a Balzers apparatus. We used JEOL's freeze-etching device (EE–FED). The dried material was transferred to a rotating table in the vacuum evaporator JEE-4C JEOL and coated with carbon and gold. All specimens were examined with a Philips scanning electron microscope PSEM 500.

For our calculation of volumes of both prepared and fresh spores, we used models made of SEM photographs (scale 2000 : 1 for *M. dispora*, and 10 000 : 1 for *N. plodiae*).

Results

The results of volume measuring of neogregarine oocysts *M. dispora* and microsporidian spores *N. plodiae* are summarized in Table 1. Volumes of prepared and fresh spores, shrinkage of the spore wall are given in this Table.

Table 1

Volumes of both neogregarine and microsporidian spores after preparation with various methods for inspection with the SEM

	Neogregarine *Mattesia dispora* Naville			Microsporidian *Nosema plodiae* Kellen et Lindergren		
method no.	spore volume (cm^3)	shrinkage in volume (%)	collapse of spore wall	spore volume (cm^3)	shrinkage in volume (%)	collapse of spore wall
1	1.95×10^{-10}	26.0	yes	4.3×10^{-12}	39.5	yes
2	1.64×10^{-10}	21.9	yes	5.4×10^{-12}	49.5	yes
3	1.98×10^{-10}	26.5	yes	3.2×10^{-12}	29.4	no
4	1.88×10^{-10}	25.0	yes	4.2×10^{-12}	38.5	no
5	1.67×10^{-10}	22.3	yes	4.2×10^{-12}	38.5	no
6	1.88×10^{-10}	25.0	yes	4.25×10^{-12}	39.0	no
7	1.57×10^{-10}	20.8	yes	6.0×10^{-12}	55.0	no
8	2.1×10^{-10}	27.1	yes	6.4×10^{-12}	58.7	no
fresh spore	7.5×10^{-10}	100	no	10.9×10^{-12}	100	no

Method No. 2 (without fixation, lyophilization) was most suitable for the neogregarine *M. dispora*. It resulted in a very smooth surface with the finnest of wrinkling (Pl. I 1) in spite of considerable changes in the volume of the spore owing to a collapse of the spore wall (the volume diminished by 1.64×10^{-10} cm^3, i.e., by 21.9% in comparison with the volume of a fresh spore). The least changes in volume were caused by method No. 8 (fixation with glutaraldehyde and OsO_4, dehydration ethanol; air-drying). However, the method caused a considerable wrinkling of the spore surface and extensive contamination brought about by a prolonged and time consuming preparation (Pl. I 2). An interesting fact observed in both fixed and unfixed spores of the neogregarine was a collapse of the spore wall, but evidently this should be ascribed to the considerably large size of these spores.

Similarly, the surface of the spore of *N. plodiae* was the smoothest after preparation with method No. 2 (without fixation; lyophilization), but changes in the volume of the spore brought about by a collapse of the spore wall were considerable (as with method No. 1) reducing the volume of the spore by 5.4×10^{-12} cm^3, .i.e., 49.5% in comparison of the volume of a fresh spore (Pl. II 3). On the other hand, the wall of fixed spores of *N. plodiae* did not collapse as did that of neogregarine, but the surface became deeply wrinkled (Pl. II 4), most distinctly with method No. 3 (fixation with OsO_4, air-drying). The least changes in the spore volume of *N. plodiae* (58.7%) followed after using method No. 8 (fixation with glutaraldehyde and OsO_4, dehydration in ethanol, air-drying), but a subsequent contamination and wrinkles in the spore surface rendered it unsuitable for a treatment of materials intended for inspection with the scanning electron microscope.

Discussion

Spores (oocysts) of neogregarines have not been examined, so far, with the SEM owing to changes in their volume and shape. The present author (Žižka 1978) was the first to examine spores of *M. dispora* in the SEM having first treated them with three different methods (freeze-drying, air-drying; OsO_4 fixation and air-drying). Åbro (1976) examined spores of eugregarine *Hoplorhynchus oligacanthus* parasitic in the gut of the zygopterans *Pyrrhosoma nymphulla* and *Enallagma cyathigerum*. This author fixed gametocysts with glutaraldehyde and formaldehyde, postfixed them with OsO_4, dehydrated in an ethanol series and dried with the critical point method. As a result of this treatment he obtained medium deformed spores with outfoldings. The

wall of the spore was collapsed, the relief low. In spite of a regular collapse of the spore wall (*M. dispora*) of the neogregarine after treatment with all our methods, there were differences in the relief of the wall and the volume of spores after treatment without different methods. In a comparison with results obtained by Åbro (1976) for spores of the eugregarine *H. oligacanthus*, we obtained a smoother surface of spores of the neogregarine *M. dispora* with our method No. 2 (without fixation; lyophilization).

Larger differences in the size of the surface relief and in the shrinkage of the volume of spores were found to occur with spores of the microsporidian *N. plodiae*. In this case, fixation prevented a collapse of the spore wall (Žižka 1977, 1978) in contrast to spores of the neogregarine *M. dispora* which always collapsed whether fixed or not. Also changes in the volume of spores were about half those occurring with spores of the neogregarine. On the other hand, the shrinkage of the volume is greater than it is with the critical point drying method (Vávra and Barker 1977, 1980). Acording to these authors an undulating surface of microsporidian spores might be associated directly with changes in the volume (*N. plodiae* and *N. bombycis*). Our results with *N. plodiae* were not in support of this their suggestion.

In conclusion we conclude that our method No. 2 (without fixation; lyophilization) was the most satisfactory for preparing spores both of the microsporidian *N. plodiae* and neogregarine *M. dispora* for inspection of their surfaces with the scanning electron microscope (minimum deformation of the spore surface and almost minimal changes of the spore volume).

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EXPLANATIONS OF PLATE I-II

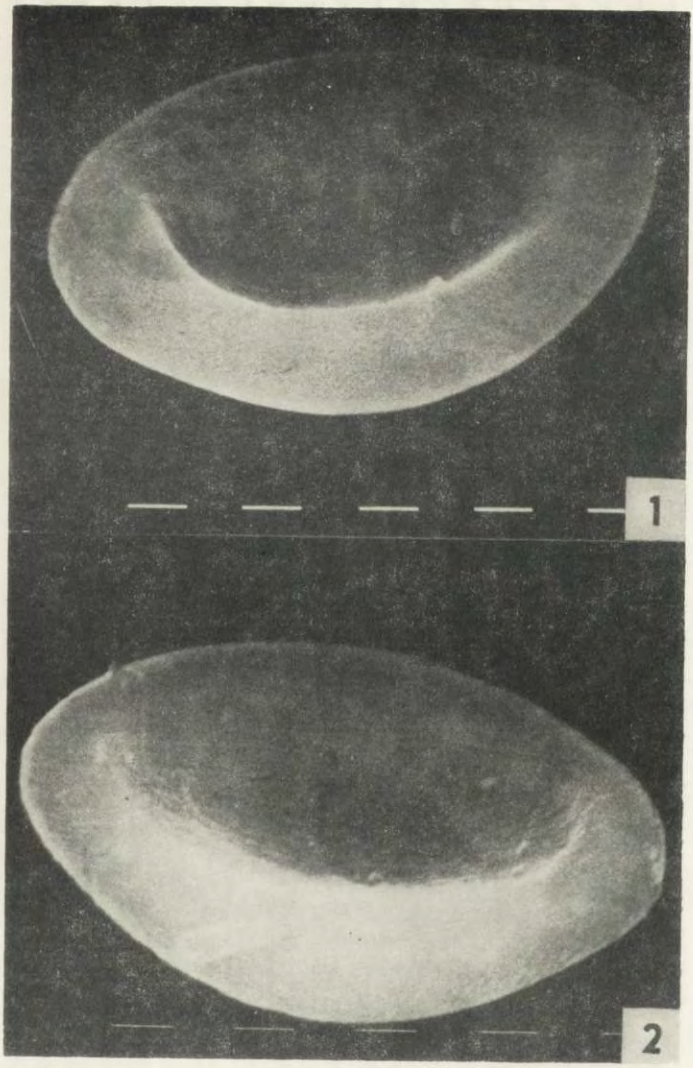
PLATE I

1: Unfixed and lyophilized spore of *M. dispora* (method No. 2) × 8000
2: Spore of *M. dispora* fixed with glutaraldehyde and OsO_4 dehydrated with ethanol and dried in the air (method No. 8) × 8000

PLATE II

3: Unfixed and lyophilized spore of *N. plodiae* (method No. 2) × 20 000
4: Spore of *N. plodiae* fixed with glutaraldehyde and OsO_4 dehydrated with ethanol and dried in the air (method No. 8) × 20 000

Microphotographs of spores (oocysts) of the neogregarine *Mattesia dispora* and spores of the microsporidian *Nosema plodiae* prepared with different methods for inspection with the scanning electron microscope. Each line on the microphotographs presents 1 μm



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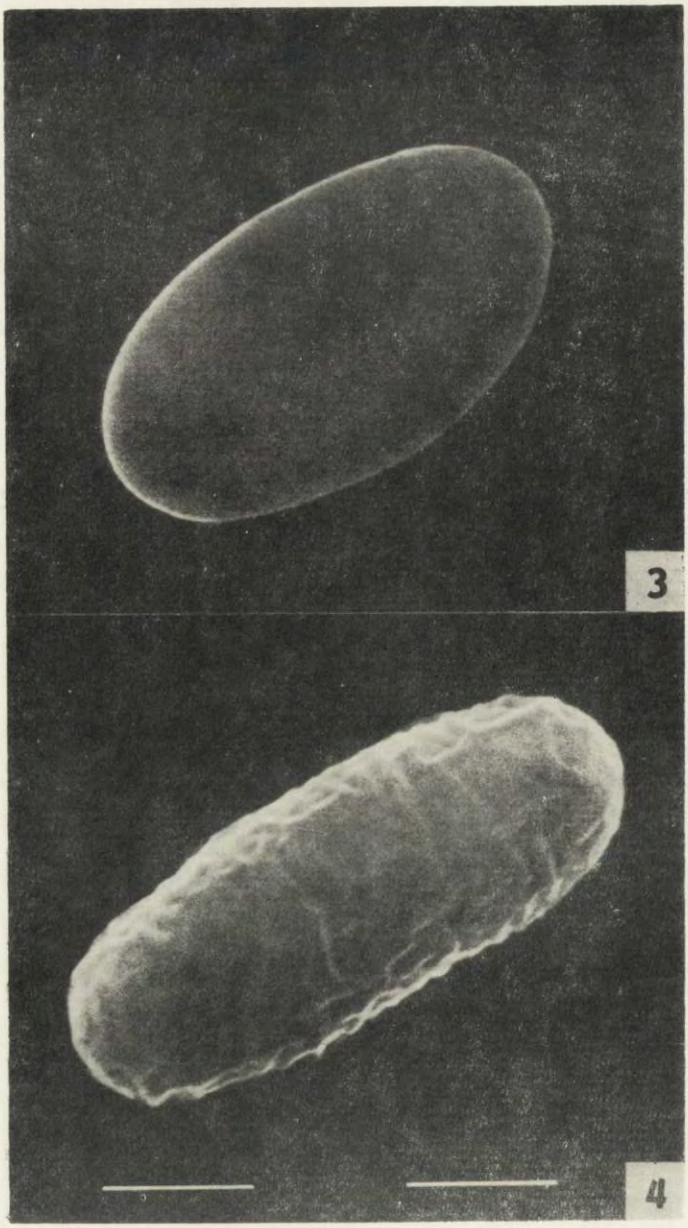

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